



PATENT
Attorney Docket No. 1352
Client Reference No. 20462-CPA-R

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Sogabe et al.

Group Art Unit: 1652

Application No. 09/940,941

Examiner: Elizabeth Slobodyansky

Filed: August 28, 2001

For: CREATINE AMIDINOHYDROLASE,
PRODUCTION THEREOF AND USE
THEREOF

DECLARATION UNDER 37 C.F.R. § 1.132 OF ATSUSHI SOGABE

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

I, Atsushi Sogabe, hereby declare that:

1. I am a citizen of Japan. I graduated in March of 1988 from the Laboratory of Applied Microbiology in the Department of Agricultural Chemistry at Shizuoka University in Shizuoka, Japan. I have been employed by Toyo Boseki Kabushiki Kaisha, the assignee with respect to the above-identified application, since April of 1988. As of July of 2003, I have been temporarily transferred to Toyobo Research Center Co., Ltd. I have been engaged in research in the fields of Enzymology and Molecular Biology. Accordingly, I am familiar with the knowledge of one of ordinary skill in the art of enzymes as of at least 1996.

2. I am one of the inventors named on U.S. Patent 6,080,553. I have read U.S. Patent 6,080,553, which I understand is related to the above-referenced reissue application.

3. U.S. Patent 6,080,553 pertains to creatine amidinohydrolases, which are described in various terms, including by reference to isoelectric point, in that patent.

4. The isoelectric point (pI) refers to the pH of a solution at which the total charges of an amphoteric electrolyte becomes 0 (i.e., neutral). The twenty different amino acids, which make up proteins, are amphoteric electrolytes having positive charges (e.g., amino group) or negative charges (e.g., carboxyl group). Each amino acid has a specific pI value. Accordingly, a protein composed of amino acids is an amphoteric electrolyte having a pI value unambiguously determinable experimentally or from the amino acid sequence of the protein.

5. U.S. Patent 6,080,553 provides the amino acid sequence of wild-type creatine amidinohydrolase derived from *Alcaligenes faecalis* in SEQ ID NO:1. Therefore, an ordinarily skilled artisan would be able to determine the pI value of the wild-type creatine amidinohydrolase by experiment and/or by calculation. The ability to do so was within the skill of an ordinarily skilled artisan since at least 1996.

6. U.S. Patent 6,080,553 describes the mutation of a wild-type creatine amidinohydrolase to create novel creatine amidinohydrolases with lower K_m values. To preserve the inherent function and the physicochemical properties of the wild-type creatine amidinohydrolase, the difference in the amino acid sequence between the source material and the mutants, or between different mutants, would have to be relatively minimal. Such small differences in the amino acid sequences of the mutants as compared to the wild-type amino acid sequence would not result in a significant difference in the pI value of the mutant creatine amidinohydrolases as compared to the wild-type enzyme.

7. One of ordinary skill in the art, reading U.S. Patent 6,080,553 in 1996, would have understood that U.S. Patent 6,080,553 describes three specific novel creatine amidinohydrolases, which can be obtained from the following deposited materials: *Escherchia coli* JM109 (pCRH273M2), *Escherchia coli* JM109 (pCRH273M1), and *Escherchia coli* JM109 (pCRH273M3). By comparison of Tables 2, 4, and 6 set forth in U.S. Patent 6,080,553, it is apparent that the majority of the physicochemical properties are conserved between the novel creatine amidinohydrolases, including the pI value.

8. One of ordinary skill in the art, reading U.S. Patent 6,080,553 in 1996, would have understood that U.S. Patent 6,080,553 also describes a group of novel creatine amidinohydrolases that include the three specific novel creatine amidinohydrolases disclosed in U.S. Patent 6,080,553.

9. If an ordinarily skilled artisan read U.S. Patent 6,080,553 in about 1996, the ordinarily skilled artisan would have recognized that these three specific novel creatine amidinohydrolases (described in U.S. Patent 6,080,553) are a representative subset of a group of novel creatine amidinohydrolases (also disclosed in U.S. Patent 6,080,553) with a shared set of physicochemical properties. The ordinarily skilled artisan also would have recognized that the pI value would be conserved among the members of this group of novel creatine amidinohydrolases as a function of conserving the function and physiological properties of the novel creatine amidinohydrolases.

10. If an ordinarily skilled artisan determined the actual pI value of the three specific novel creatine amidinohydrolases described in U.S. Patent 6,080,553 and found the actual pI value to be different from the pI value reported in U.S. Patent 6,080,553 for those three specific novel creatine amidinohydrolases, the ordinarily skilled artisan nevertheless would have understood that the pI value characterizing the group of novel creatine amidinohydrolases would be about the same as the actual determined pI value characterizing the three specific novel creatine amidinohydrolases that are members of that group. One of ordinary skill in the art would have had that understanding in 1996 and would have that same understanding today. Reading U.S. Patent 6,080,553 either in 1996 or today, one of ordinary skill in the art would believe that the inventors listed on U.S. Patent 6,080,553 had possession of the group of creatine amidinohydrolases with a pI value that was the same as the pI value of the three specific novel creatine amidinohydrolases described in U.S. Patent 6,080,553, even if the actual pI value for these three specific novel creatine amidinohydrolases differed from the pI value reported in U.S. Patent 6,080,553.

11. The pI values of the creatine amidinohydrolases described in Examples 4, 5, and 6 of U.S. Patent 6,080,553 were determined experimentally in the following manner. The three deposited bacterial strains that produce the creatine amidinohydrolases described in Examples 4, 5, and 6 of U.S. Patent 6,080,553 (i.e., *Escherichia coli* JM109 (pCRH273M1) deposited as FERM BP-5374, *Escherichia coli* JM109 (pCRH273M2) deposited as FERM BP-5375, and *Escherichia coli* JM109 (pCRH273M3) deposited as FERM BP-5376, respectively) were obtained from the International Patent Organism Depository (IPOD), a Japanese International Depository Authority. Each bacterial strain was cultured in an L-broth (1.0% polypeptone, 0.5% yeast extract, 0.5% sodium chloride,

pH 7.4) containing 100 mg/mL ampicillin and 1.5% agar at 30° C for 24 hr. For a preculture, a part of the bacterial cells grown was transferred to 5 mL of an L-broth (pH 7.4) containing 100 mg/ml ampicillin with an inoculating loop, and cultured with shaking at 30° C for 16 hr. Then, the obtained bacterial suspension (0.5 mL) was transferred to 50 mL of a creatine amidinohydrolase-producing medium I (1.2% polypeptone, 2.4% yeast extract, 0.4% glycerol, 1.25% di-potassium hydrogen phosphate, 0.23% potassium dihydrogen phosphate, and 200 mg/ml ampicillin), and cultured with shaking at 30° C for 22 hr.

12. After the completion of the culture in the creatine amidinohydrolase-producing medium I, each bacterial suspension was centrifuged to recover bacterial cells, which were suspended in 20 mM potassium phosphate buffer (pH 7.5) and sonicated to extract creatine amidinohydrolase. The creatine amidinohydrolase was purified by polyethyleneimine treatment, ammonium sulfate fractionation, a heat treatment, DEAE-sepharose exchange column chromatography, and Octyl-Sepharose column chromatography. The purified creatine amidinohydrolase from each bacterial suspension gave a single band with SDS polyacrylamide gel electrophoresis.

13. *Alcaligenes faecalis* strain TE3581 described in Reference Example 1 of U.S. Patent 6,080,553 was cultured in an L-broth (1.0% polypeptone, 0.5% yeast extract, 0.5% sodium chloride, pH 7.4) containing 1.5% agar at 30° C for 47 hr. For preculture, a part of the bacterial cells grown was transferred to 5 mL of an L-broth (pH 7.4) with an inoculating loop and cultured with shaking at 30° C for 16 hr. Then, the obtained bacterial suspension (5 mL) was transferred to 500 mL of a creatine amidinohydrolase-producing medium II (0.65% creatinine, 1.2% polypeptone, 2.4% yeast extract, 0.4% glycerol, 1.25% di-potassium hydrogen phosphate, and 0.23% potassium dihydrogen phosphate), and cultured with shaking at 30° C for 48 hr.

14. After the completion of the culture in the creatine amidinohydrolase-producing medium II, the bacterial suspension was centrifuged to recover bacterial cells, which were suspended in 20 mM potassium phosphate buffer (pH 7.5) and sonicated to extract creatine amidinohydrolase. The creatine amidinohydrolase was purified by polyethyleneimine treatment, ammonium sulfate fractionation, a heat treatment, DEAE-sepharose exchange column chromatography, and Octyl-Sepharose column

chromatography. The purified creatine amidinohydrolase gave a single band with SDS polyacrylamide gel electrophoresis.

15. The pI value of each purified creatine amidinohydrolase described above, as well as CRH-211, a creatine amidohydrolase derived from *Actinobacillus sp.* (commercially available from Toyo Boseki K.K.), was determined using Fast System (Amersham Bioscience). pI Calibration Kit 3-10 (Amersham Bioscience) and pI Calibration Kit 2.5-6.5 (Amersham Bioscience) were used for the pI markers.

16. The pI values of the above-identified purified creatine amidinohydrolases were determined to be approximately 4.5, as reported in Figure 1 (see Attachment A).

17. Additionally, the pI values of creatine amidinohydrolases isolated from various other strains are known in the art to be approximately 4.5. For example, the pI value of the creatine amidinohydrolase from *Actinobacillus sp.* is 4.6 (see page 75 of "Creatine Amidinohydrolase from *Actinobacillus sp.*," Toyobo Co., Ltd. Catalog (Attachment B)). The pI value of the creatine amidinohydrolase of *Pseudomonas sp.* is 4.7 (see page 512, second column, last paragraph, of Yoshimoto et al., *Arch. Biochem. Biophys.*, 177, 508-515 (1976) (Attachment C); and page 1 of "Creatine Amidinohydrolase from *Pseudomonas sp.*," Toyobo Co., Ltd. Catalog (Attachment D)). The pI value of the creatine amidinohydrolase of *Pseudomonas sp.* (expressed in recombinant *E. coli*) is 4.8 (see page 48 of "Creatinase, *Pseudomonas* species, recombinant," Roche Molecular Biochemicals 1999/2000 (Attachment E)). The pI value of the creatine amidinohydrolase of *Arthrobacter sp.* is 4.3 (see Abstract; page 3, first column, lines 2-12 and 17-30; and page 5, second column, lines 25-28, of JP 10-257890 (Attachment F)). The pI value of the creatine amidinohydrolase of *Paracoccus sp.* is 4.1 (see page 5, second column, line 37, of JP 7-274961 (Attachment G)). The pI value of the creatine amidinohydrolase of *Bacillus sp.* is 4.9 (see column 4, lines 10-12, of U.S. Patent 4,420,562 (Attachment H)). The pI value of the creatine amidinohydrolase of *Alcaligenes sp.* is 4.7 (see page 2157, penultimate paragraph, of Matsuda et al., *Chem. Pharm. Bull.*, 34(5), 2155-2160 (1960) (Attachment I)).

18. These results demonstrate that the pI values of the creatine amidinohydrolases disclosed in U.S. Patent 6,080,553 are about 4.5, and also that the pI values of creatine amidinohydrolases from different strains are about 4.5. Thus, one of

In re Appln. of Sogabe et al.
Application No. 09/940,941

that the named inventors of U.S. Patent 6,080,553 had possession of such a group of novel creatine amidinohydrolases.

19. I hereby declare that all statements made herein of my own knowledge are true, that all statements made on information and belief are believed to be true, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date:

2003 11 6

Atsushi Sogabe

Atsushi Sogabe

Applicant Initiated Interview Request Form

Application No.: 09/940,941 First Named Applicant: Sogabe et al.
Examiner: E. Slobodyansky Art Unit: 1652 Status of Application: RCE filed November 6, 2003

Tentative Participants:

- (1) John Kilyk, Jr. (attorney of record) (2) Atsushi Sogabe (inventor)
(3) Satoshi Murayama (assignee representative) (4) Katsuhiko Nose (assignee representative)
(5) Hiroshi Shibano (assignee representative) (6) Kazumi Yamamoto (assignee representative)
(7) Supervisory Primary Examiner

Proposed Date of Interview: December 1, 2003 Proposed Time: 10 AM

Type of Interview Requested:

- (1) ☐ Telephonic (2) ☒ Personal (3) ☐ Video Conference

Exhibit To Be Shown or Demonstrated: ☐ YES ☒ NO

If yes, provide brief description: _____

Issues To Be Discussed

Issues (Rej., Obj., etc.)	Claims/ Fig.#s	Prior Art	Discussed	Agreed	Not Agreed
(1) <u>112, first paragraph</u>	<u>24-42</u>	_____	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
(2) <u>112, second paragraph</u>	<u>27, 29, 31, 33-37</u>		<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
(3) <u>102(b)</u>	<u>24, 28</u>	<u>JP 62-099182</u>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
(4) <u>Claim Amendments and New Claims</u>			<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

☐ Continuation Sheet Attached

Brief Description of Arguments to be Presented:

Discussion of "Response to Office Action" dated May 1, 2003, "Advisory Action" dated May 22, 2003, and "Supplemental Response to Office Action" and "Declaration Under 37 C.F.R. § 1.132 of Atsushi Sogabe" filed herewith.

An interview was conducted on the above-identified application on _____.

NOTE:

This form should be completed by applicant and submitted to the examiner in advance of the interview (see MPEP § 713.01).

This application will not be delayed from issue because of applicant's failure to submit a written record of this interview. Therefore, applicant is advised to file a statement of the substance of this interview (37 CFR 1.133(b)) as soon as possible.

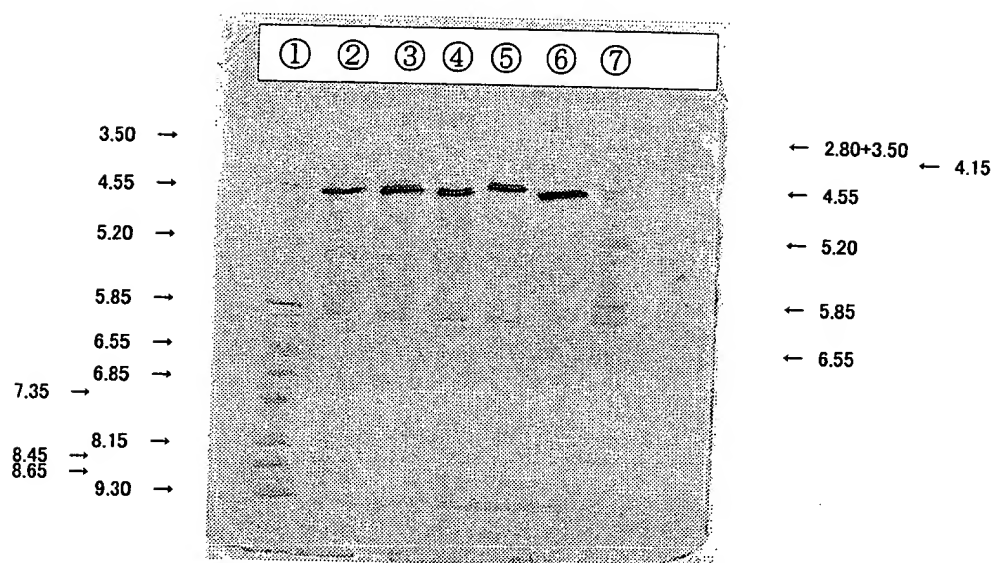
(Applicant/Applicant's Representative Signature)

(Examiner/SPE Signature)

This collection of information is required by 37 CFR 1.133. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

If you need assistance in completing this form, call 1-800-PTO-9199 and select option 2.

Fig. 1



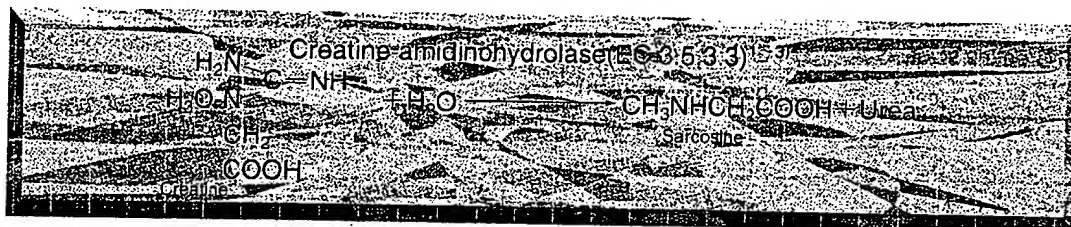
- Lane ①: pI Marker (pI Calibration Kit 3-10)
 Lane ②: creatine amidinohydrolase from *Alcaligenes faecalis* TE3581
 Lane ③: creatine amidinohydrolase from *E. coli* JM109 (pCRH273M1)
 Lane ④: creatine amidinohydrolase from *E. coli* JM109 (pCRH273M2)
 Lane ⑤: creatine amidinohydrolase from *E. coli* JM109 (pCRH273M3)
 Lane ⑥: creatine amidinohydrolase from *Actinobacillus* sp.
 Lane ⑦: pI Marker (pI Calibration Kit 2.5-6.5)

TOYOBO ENZYMES
(Diagnostic Reagent Grade)

Attachment B

CREATINE AMIDINOHYDROLASE

from Actinobacillus sp.



PREPARATION and SPECIFICATION

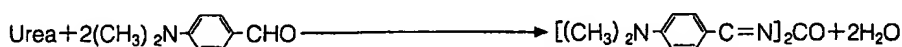
Appearance	: White amorphous powder, lyophilized	
Activity	: Grade II 6.0U/mg-solid or more (containing approx. 50% of stabilizers)	
Contaminants	: NADH oxidase $\leq 5.0 \times 10^{-2}\%$ Catalase $\leq 2.0\%$	
Stability	: Stable at 0–5°C in a desiccator	(Fig.1)
Stabilizers	: Sugars, EDTA	

PROPERTIES

Molecular weight	: approx. 100,000	
Isoelectric point	: 4.6 ± 0.1	
Michaelis constant	: $1.9 \times 10^{-2} \text{M}$ (Creatine)	
Structure	: 2 subunits per mol of enzyme	
Inhibitors	: Cu^{++} , Hg^{++} , Ag^{+}	
Optimum pH	: 8.0	(Fig.3)
Optimum temperature	: 40°C	(Fig.4)
pH Stability	: pH 5.5–9.0 (25°C, 16hr)	(Fig.5)
Thermal stability	: below 50°C (pH 7.5, 30min)	(Fig.6)
Effect of various chemicals	: (Table 1)	

APPLICATIONS

This enzyme is useful for enzymatic determination of creatine and creatinine when coupled with sarcosine dehydrogenase (or sarcosine oxidase) and formaldehyde dehydrogenase in clinical analysis.⁴⁾

ASSAY**Principle:**

The appearance of yellow dye formed by condensation of urea and p-dimethylaminobenzaldehyde (DAB) (Ehrlich reaction) is measured at 435nm by spectrophotometry.

Unit definition:

One unit causes the formation of one micromole of yellow dye per minute under the conditions described below.

Method:**Reagents**

- A. Creatine solution : 0.1M [1.49g creatine (Merck)/100ml of 50mM phosphate buffer, pH 7.5] (Should be prepared fresh)
- B. DAB solution : Dissolve 2.0g of DAB in 100ml of dimethylsulfoxide and, to this solution, add 15ml of conc. HCl solution.
- C. Enzyme diluent : 50mM Phosphate buffer, pH 7.5

Procedure

1. Pipette 0.9ml of the substrate solution (A) into a test tube and equilibrate at 37°C for about 5 minutes.
2. Add 0.1ml of the enzyme solution* and mix.
3. After exactly 10 minutes at 37°C, add 2.0ml of DAB solution (B) to stop the reaction.
4. Incubate at 25°C for 20 minutes.
5. Measure the optical density at 435nm against water (OD test).

Concentration in assay mixture	
Phosphate buffer	50mM
Creatine	90mM

At the same time, prepare the blank by first mixing the substrate solution with 2.0ml of DAB solution after a 10 min-incubation at 37°C, followed by the addition of the enzyme solution, and carry out the same procedure as test (procedure 4 and 5) (OD blank).

- * Dissolve the enzyme preparation in ice-cold enzyme diluent (C) and dilute to 1.0–4.0 U/ml with the same buffer, immediately before assay.

Calculation

Activity can be calculated by using the following formula :

$$\text{Volume activity (U/ml)} = \frac{\Delta \text{OD}(\text{OD test} - \text{OD blank}) \times V_t \times df}{0.321 \times 1.0 \times t \times V_s} = \Delta \text{OD} \times 9.35 \times df$$

$$\text{Weight activity (U/mg)} = (\text{U/ml}) \times 1/C$$

V_t : Total volume (3.0ml)

V_s : Sample volume (0.1ml)

0.321 : Millimolar extinction coefficient of yellow dye (cm/micromole)

1.0 : Light path length (cm)

t : Reaction time (10 minutes)

df : Dilution factor

C : Enzyme concentration in dissolution (c mg/ml)

REFERENCES

- 1) D.Tsuru; *Nucleic Acid and Amino Acids*, 35, 31 (1977).
- 2) T.Yoshimoto, I.Oka and D.Tsuru; *Arch.Biochem.Biophys.*, 177, 508 (1976).
- 3) T.Yoshimoto, I.Oka and D.Tsuru; *J.Biochem.*, 79, 1381 (1976).
- 4) D.Tsuru; *Rinsho Kensa*, 22, 1331 (1978).

Table 1. Effect of Various Chemicals on Creatine amidinohydrolase

[The enzyme dissolved in 50mM Tris-HCl buffer, pH 7.5 (80 U/ml) was incubated at 25°C for 30 minutes with each chemical.]

Chemical	Concn. (mM)	Residual activity	Chemical	Concn. (mM)	Residual activity
None	—	100%	NaF	1.0	105 %
Metals	1.0	100	PCMB	0.33	13.3
AsCl ₃	1.0	107	MIA	1.0	106
BiCl ₃	1.0	109	IAA	1.0	103
MeCl	1.0	107	NaN ₃	10	106
NaCl	1.0	107	o-Phenanthroline	1.0	108
CaCl ₂	1.0	108	Hydroxylamine	1.0	105
FeCl ₃	1.0	104	NEM	10	10.3
CoCl ₂	1.0	88	Triton X-100	0.5%	94
HgCl ₂	1.0	108	Brij 35	0.5%	103
PbCl ₂	1.0	102	Tween 20	0.5%	100
AgCl	1.0	27	Span 20	0.5%	106
ZnSO ₄	1.0	97	Na-cholate	0.5%	103
CuSO ₄	1.0	140	SDS	0.25%	102
FeCl ₃ ·xH ₂ O	1.0	103	DAC	0.5%	1.7
Ca(NO ₃) ₂	1.0	2.5			
EDTA	20	99			
α,α'-Dipyridyl	1.0	100			

Ac, CH₃CO; EDTA, Ethylenediaminetetraacetate; PCMB, p-Chloromercuribenzoate; MIA, Monoiodoacetate; IAA, Iodoacetamide; NEM, N-Ethylmaleimide; SDS, Sodium dodecyl sulfate; DAC, Dimethyl-benzyl-alkyl-ammonium chloride

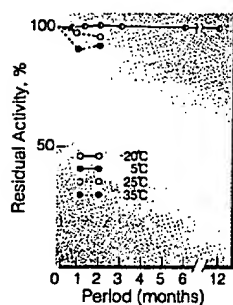


Fig.1. Stability (Powder form)
(kept under dry conditions)

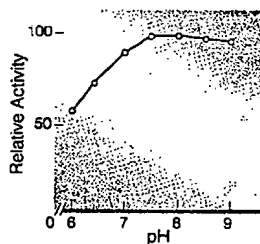


Fig.3. pH-Activity
(37°C, 10min-reaction in 50mM K-phosphate buffer)

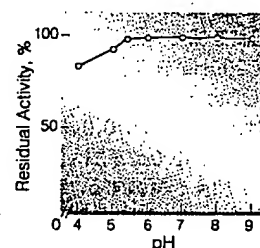


Fig.5. pH-Stability
(25°C, 16hr-treatment with 50mM buffer solution: pH4.0-5.5, Acetate pH6.0-9.0, K-phosphate)

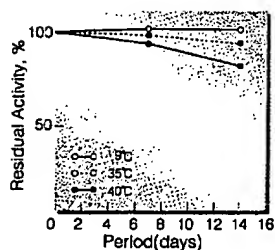


Fig.2. Stability (Liquid form)
(In 50 mM K-phosphate buffer, pH7.5)

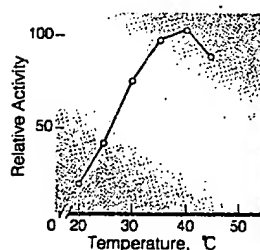


Fig.4. Temperature activity
(10min-reaction in 50mM K-phosphate buffer, pH7.5)

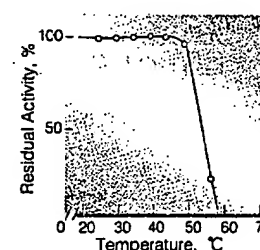
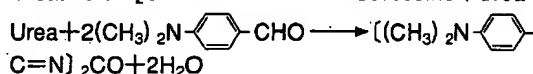


Fig.4. Thermal stability
(30min-treatment with 50mM K-phosphate buffer, pH7.5)

活性測定法 (Japanese)

1. 原理



生成した尿素のp-ジメチルアミノベンズアルデヒド (DAB) との縮合 (Ehrich 反応) 生成物 (黄色色素) を比色定量する。

2. 定義

下記条件下で1分間に1マイクロモルの黄色色素を生成する酵素量を1単位 (U) とする。

3. 試薬

- A. 0.1M クレアチン溶液 [1.49g のクレアチン (Merck 製) を 50mM リン酸緩衝液 pH7.5 に溶解し、100ml とする] (用時調製)
- B. DAB 溶液 (2.0g の p-ジメチルアミノベンズアルデヒドを 100ml のジメチルスルホキシドに溶解させた後、濃塩酸 15ml を加える)

酵素溶液：酵素標品を予め氷冷した 50mM リン酸緩衝液、pH7.5 で溶解し、分析直前に同緩衝液で 1.0~4.0U/ml に希釈する。

4. 手順

- ① 試験管に基質溶液 (A) 0.9ml を採り、37℃ で約 5 分間予備加温する。
- ② 酵素溶液 0.1ml を加え、反応を開始する。
- ③ 37℃ で正確に 10 分間反応させた後、DAB 溶液 (B) 2.0ml を加えて反応を停止させる。
- ④ 25℃ で 20 分間放置後、435nm における吸光度を測定する (OD test)。
- ⑤ 盲検は基質溶液 (A) 0.9ml を 37℃ で 10 分間放置後、DAB 溶液 (B) 2.0ml を加えて混和し、次いで酵素溶液 0.1ml を加えて調製する。以下同様に 25℃ で 20 分間放置後吸光度を測定する (OD blank)。

5. 計算式

$$\text{U/ml} = \frac{\Delta \text{OD} (\text{OD test} - \text{OD blank}) \times 3.0 (\text{ml}) \times \text{希釈倍率}}{0.321 \times 1.0 \times 10 (\text{分}) \times 0.1 (\text{ml})}$$

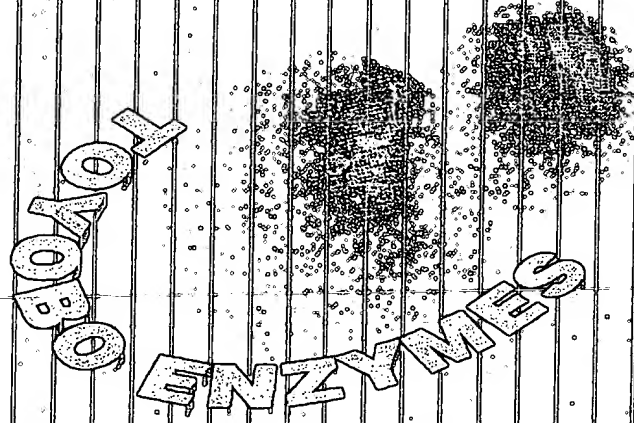
$$= \Delta \text{OD} \times 9.35 \times \text{希釈倍率}$$

$$\text{U/mg} = \text{U/ml} \times 1 / C$$

0.321 : 黄色色素のミリモル分子吸光係数
(cm/micromole)

1.0 : 光路長 (cm)

C : 溶解時の酵素濃度 (c mg/ml)



A1827K

Printed in Japan
July 1, 2000

Rev 1-2

Creatine Amidinohydrolase of *Pseudomonas putida*: Crystallization and Some PropertiesTADASHI YOSHIMOTO, IMAO OKA,¹ AND DAISUKE TSURU

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Creatine amidinohydrolase (EC 3.5.3.3, creatinase) of *Pseudomonas putida* var. *naraensis* C-83 was purified by column chromatography on sarcosine-hexamethylenediamine-Sepharose and Sephadex G-200 and then crystallized in the presence of ammonium sulfate. The purified preparation appeared homogeneous on disc gel electrophoresis and ultracentrifugal analysis. It was most active at pH 8 and showed a K_m value of 1.33 mM for creatine. Estimation of the molecular weight by the meniscus depletion method yielded a value of 94,000. A value of 47,000 was obtained, however, by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, suggesting that the enzyme is composed of two subunits. Inhibition experiments suggested that a sulphydryl group is closely related to the creatinase activity.

In the previous paper (1), we reported that *Pseudomonas putida* var. *naraensis* C-83 inducibly produces three intracellular enzymes involved in creatinine metabolism, i.e., creatinine amidohydrolase (EC 3.5.2, creatininase), creatine amidinohydrolase (EC 3.5.3.3, creatinase), and sarcosine dehydrogenase (EC 1.5.99.1). By the action of these enzymes, creatinine is converted to sarcosine via creatine and further to glycine (1). The same type of metabolic pathway for creatinine has previously been deduced in the other two species of *Pseudomonas*, *P. aeruginosa* (2-4) and *P. ovalis* (5, 6). However, the details of the properties of the enzymes involved have remained unclear. Kaplan and his co-workers (7, 8) recently reported the purification and some enzymatic properties of creatininase and creatinase from *Arthrobacter ureafaciens*, but the preparations obtained were still heterogeneous.

We attempted to purify these three enzymes from cell-free extracts of *P. putida* grown in a creatinine-enriched medium (1) and succeeded in obtaining a crystalline creatinase and characterizing its properties. The present paper deals with the pu-

rification, the crystallization, and the details of the properties of the creatinase from *P. putida*. This is the first report that describes details of the crystallization method and properties of the crystalline creatinase from *P. putida*. A brief communication of the purification procedure appeared as a preliminary note (9).

MATERIALS AND METHODS

Strain used and culture conditions. *P. putida* var. *naraensis* C-83 was cultivated at 37°C aerobically in a medium containing 0.5% creatinine, 0.1% yeast extract, 0.025% malt extract, and 0.05% NH_4Cl in 0.1 M potassium phosphate buffer, pH 7.4 (1). After cultivation for 22 h, the cells were harvested by centrifugation and washed twice with 10 mM phosphate buffer, pH 7.0.

Enzyme activity assay. The activities of creatininase, creatinase, and sarcosine dehydrogenase were assayed as described previously (1), and one unit of each activity was defined as the enzyme quantity that decomposes 1 μmol of substrate per minute under the assay conditions. Specific activity was expressed as units per milligram of protein.

Preparation of sarcosine-HM-Sepharose.² HM-

² Abbreviations used: HM, hexamethylenediamine; NTB, nitrotertrazolum blue; ORD, optical rotatory dispersion; PCMB, *p*-chloromercuribenzoate; PMS, phenazine methosulfate; SDS, sodium dodecyl sulfate; BPB, bromphenol blue.

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Sephacrose was prepared from CNBr-activated Sepharose 4B and hexamethylenediamine according to the method of Cuatrecasas (10). This was coupled with sarcosine in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide as follows. HM-Sepharose (50 ml as packed gel) was suspended in an equal volume of water containing 2 mmol of sarcosine and 10 mmol of the water-soluble carbodiimide was added to the suspension. The reaction mixture was gently stirred at room temperature for 20 h, during which the pH was maintained to 4.75 with 2 N HCl. The resulting sarcosine-HM-Sepharose was washed successively with water, 0.1 M NaHCO₃, and water. The preparation was found to contain about 20 μ mol of sarcosine per gram of dried gel as judged by amino acid analysis of the acid hydrolysate.³

Disc electrophoresis. This was carried out by the use of a pH 8.4 gel according to the method of Davis (11). Enzyme preparation (20–50 μ g in 10 μ l) was loaded on a gel and a current of 2 mA per tube was supplied for 1.5 h in the cold. Protein was stained with 1% Amidoblau. Specific staining of the creatinase fraction in the gel was carried out by coupling the creatinase reaction with the sarcosine dehydrogenase system as follows. The gels were soaked, after electrophoresis, in 10 ml of 50 mM phosphate buffer, pH 8.0, containing 0.1 M creatine, 50 μ g of phenazine methosulfate (PMS), 50 μ g of nitroterrazolium blue (NTB), and 10 units of sarcosine dehydrogenase at 37°C for 1 h in the dark. This treatment had led to the formation of diformazan (reduced nitroterrazolium blue), whose blue color showed the position of creatinase.

SDS-polyacrylamide gel electrophoresis. The method of Weber and Osborn (12) was adopted with a slight modification. The lyophilized enzyme (200 μ g) was dissolved in 0.5 ml of 10 mM phosphate buffer, pH 7.0, containing 25% glycerol, 1% SDS, and 0.01% BPB with or without 0.5% 2-mercaptoethanol. After incubation at 37°C overnight, 100- μ l aliquots of the sample and marker proteins were each subjected to electrophoresis on 10% polyacrylamide gels at a constant current of 8 mA per gel for 3 h with a positive electrode in the lower chamber filled with 0.1 M phosphate buffer containing 0.1% SDS. The gels were stained with 0.25% Coomassie brilliant blue R-250 and destained by washing with a mixture of acetic acid-methanol-water (35:105:315) for 1 day. The molecular weight of the sample was estimated by comparison with parallel runs of the standard marker proteins, aldolase, and phosphorylase a from rabbit muscle and beef liver catalase.

Isoelectric focusing experiment. This was done by the method of Vesterberg and Svensson as given in Matsuo and Horio (13) with an LKB apparatus (Sweden). Five milliliters of the enzyme solution

was dialyzed against 0.2% carrier ampholyte (pH 4–6) for 20 h, applied to a column (110 ml in volume) with a sucrose density gradient and then subjected to isoelectric focusing with a current of 3 mA for 45 h at 4°C.

Ultracentrifugal analysis. The enzyme was dialyzed against 50 mM acetate buffer, pH 5.8, containing 0.1 M NaCl at 4°C for 40 h. The dialyzed enzyme solution and the outer solution were used for the ultracentrifugal experiments which were performed at 20°C by using a Beckman Model E Ultracentrifuge equipped with Schlieren and interference optics.

Optical rotatory dispersion. This was measured using a JASCO ORD recorder Model UV-5. A quartz cell of 20-mm optical path with a 0.5 to 1.0% protein sample was used for the measurements over the range from visible to the near-ultraviolet region (300–600 nm). A value of 113.3 was used as the mean residue molecular weight, M_0 , which was calculated from the amino acid composition. The values of a_0 and b_0 were calculated by the method of Urnes and Doty (14) and the α -helix content of the enzyme was estimated from the b_0 value assuming that a polypeptide composed completely of α -helix structure gave a b_0 value of –630.

Amino acid composition. Amino acid composition of the enzyme was determined by the method of Spackman *et al.* (15) with a Nippon Denshi JLC-6AH automatic amino acid analyzer. Contents of half-cystine and tryptophan were determined by the methods of Moore (16) and Goodwin and Morton (17), respectively.

The sugar content of the enzyme was measured by the phenol-sulfuric acid method (18).

Chemicals used. Aldolase and phosphorylase A from rabbit muscle and beef liver catalase were purchased from Boehringer-Mannheim, Germany. Creatine, creatinine, sarcosine, dithiothreitol, PCMB, and sodium tetrathionate were from Nakarai Kagaku Co., Kyoto, Japan. PMS and NTB were products of Dojin Chemical Laboratories Co., Kumamoto, Japan, and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide was prepared at the Peptide Center of Osaka University, Osaka, Japan. Sepharose 4B and Sephadex G-200 were supplied from Pharmacia Fine Chemicals, Sweden.

RESULTS AND DISCUSSION

Purification and Crystallization of Creatinase from P. putida var. naraensis C-83

All of the procedures were carried out at 4°C. The washed cells, 100 g wet weight, were suspended in 500 ml of 50 mM phosphate buffer, pH 7.0, mixed with an equal volume of glass beads and disrupted by a Vibrogen Cell Mill (Edmund Bühler, Germany) for 10 min. The suspension of the

³ Most of sarcosine was converted to glycine during acid hydrolysis.

disrupted cells was centrifuged at 10,000 rpm for 20 min, and ammonium sulfate was added to the cell-free supernatant to give a final concentration of 0.8 saturation. After 3 h, the resulting precipitate was collected by centrifugation, dissolved in 50 mM phosphate buffer, pH 7.2, and then dialyzed against the same buffer for 2 days. The dialysate was divided into five portions and each of them was applied to a column (3 × 20 cm) of sarcosine-HM-Sephadex which had previously been equilibrated with 50 mM phosphate buffer, pH 7.2, and the enzymes adsorbed were eluted with a linear gradient system of NaCl concentration. The reservoir and the mixing chamber contained 1 liter each of 50 mM phosphate buffer, pH 7.2, with and without 1 M NaCl. A typical chromatogram is illustrated in Fig. 1. The three enzymes related to creatinine metabolism were all adsorbed onto the column and creatinase was eluted with the buffer containing 70 mM NaCl, whereas sarcosine dehydrogenase and creatininase were recovered at 0.4 and 0.6 M NaCl concentrations, respectively. Creatinase fractions

from five separate columns were combined, and ammonium sulfate was added to the enzyme solution to give 0.8 saturation. The precipitate was collected by centrifugation, dialyzed against 50 mM phosphate buffer, pH 7.2, and rechromatographed as above. The creatinase was again salted out with ammonium sulfate, dialyzed as above, and then subjected to gel filtration on a column (3 × 120 cm) of Sephadex G-200 equilibrated with the buffer used above. An almost symmetric protein peak with a constant specific activity was obtained. Creatinase fractions were combined and concentrated by using a collodion bag (MS Instrument Co., Osaka, Japan) and the concentrate ($A_{280\text{ nm}} = 29$) was dialyzed against 50 mM phosphate buffer, pH 7.2, for 1 week, during which a saturated solution of ammonium sulfate was added dropwise to a final concentration of 0.25 saturation. After the enzyme solution remained in a refrigerator for 2 to 5 additional days, the crystalline creatinase appeared in a yield of about 6% of the original total activity. Figure 2 shows a microphotograph of crystalline creatinase. The specific activity was increased more than 60-fold by this purification procedure. A summary of the enzyme purification is shown in Table I.

Figure 3 shows disc electrophoretic profiles of the purified creatinase. A single protein band was observed, which also showed creatinase activity as checked by the enzyme staining method described under Materials and Methods. A symmetrical Schlieren pattern was observed throughout the ultracentrifugal sedimentation run of the purified enzyme (Fig. 4). Neither creatininase nor sarcosine dehydrogenase activities were detected in the purified creatinase preparation.

Enzymatic Properties

The enzyme was most active at pH 8 and stable between pH values of 6 and 8. Optimal temperature was at 30°C during 30 min of reaction, and half of the original activity remained after incubation at 45°C and pH 7.4 for 30 min. The K_m and k_{cat} (V/E_0) values of the creatinase for creatine were determined to be 1.33 mM and 0.246

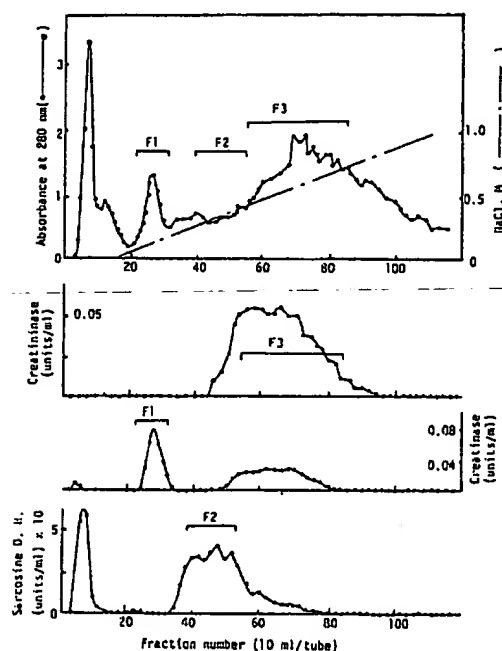


FIG. 1. Chromatogram of creatininase, creatinase, and sarcosine dehydrogenase on sarcosine-HM-Sephadex column. See the text for the experimental details. Sarcosine D.H., sarcosine dehydrogenase.

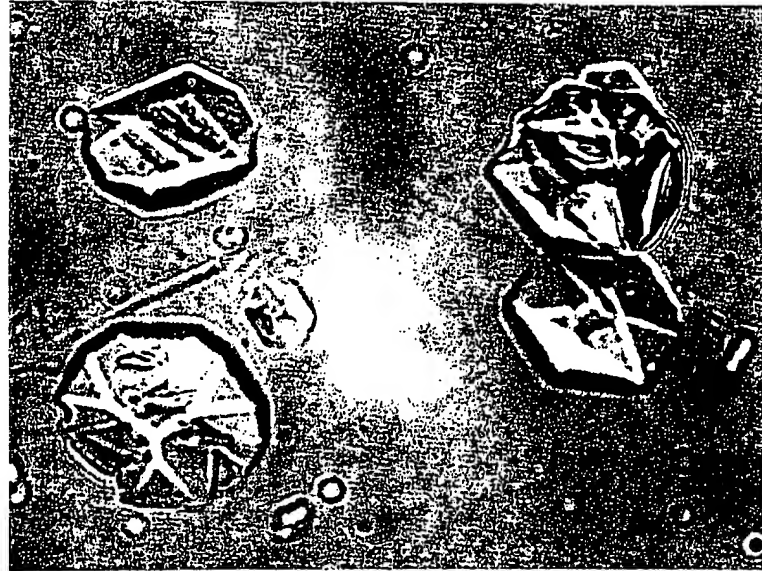


FIG. 2. Micrograph of crystalline creatinase.

TABLE I
PURIFICATION OF CREATINASE FROM *P. putida*

Fraction	Volume (ml)	Protein concentration (mg/ml) ^a	Total protein (g)	Activity (units/ml)	Total activity	Specific activity	Recovery (%)
Culture broth	25,000 ^b	—	—	0.019	475	—	100
Cell-free extract	1,500	100.0	150.0	0.200	300	0.002	63
Salting out and dialysis	430	97.0	41.0	0.388	166.8	0.004	35
Elution from sarcosine-HM-Sepharose	1,200	0.45	0.54	0.045	54.0	0.100	11.4
Salting out and dialysis	20	18.40	0.368	1.930	38.6	0.105	8.1
Gel filtration on Sephadex G-200	85	3.62	0.308	0.420	35.7	0.116	7.5
Crystalline enzyme	—	—	0.222	—	28.5	0.128	6.0

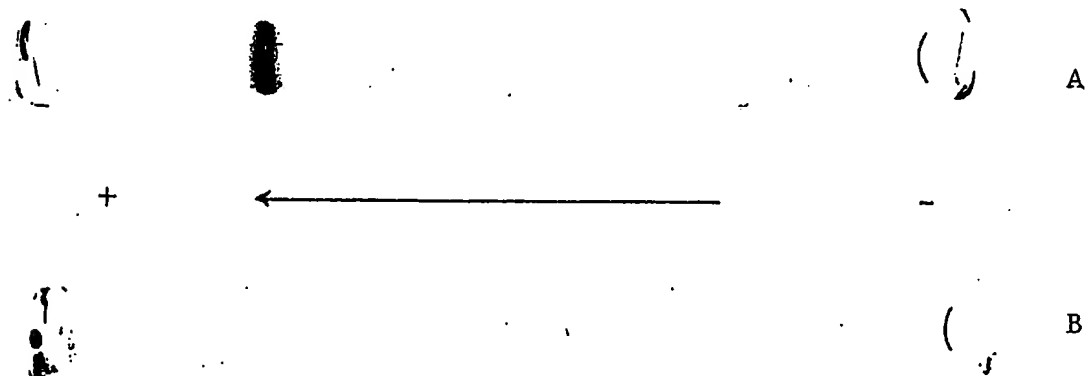
^a Estimated spectrophotometrically assuming that $A_{1\text{cm}}^{1\%}$ at 280 nm for protein is 11.1.^b Contained about 300 g cells wet weight.

FIG. 3. Disc gel electrophoretic patterns of purified creatinase. (A) Stained with 1% Amidoschwarz; (B) detected by the enzyme staining method.

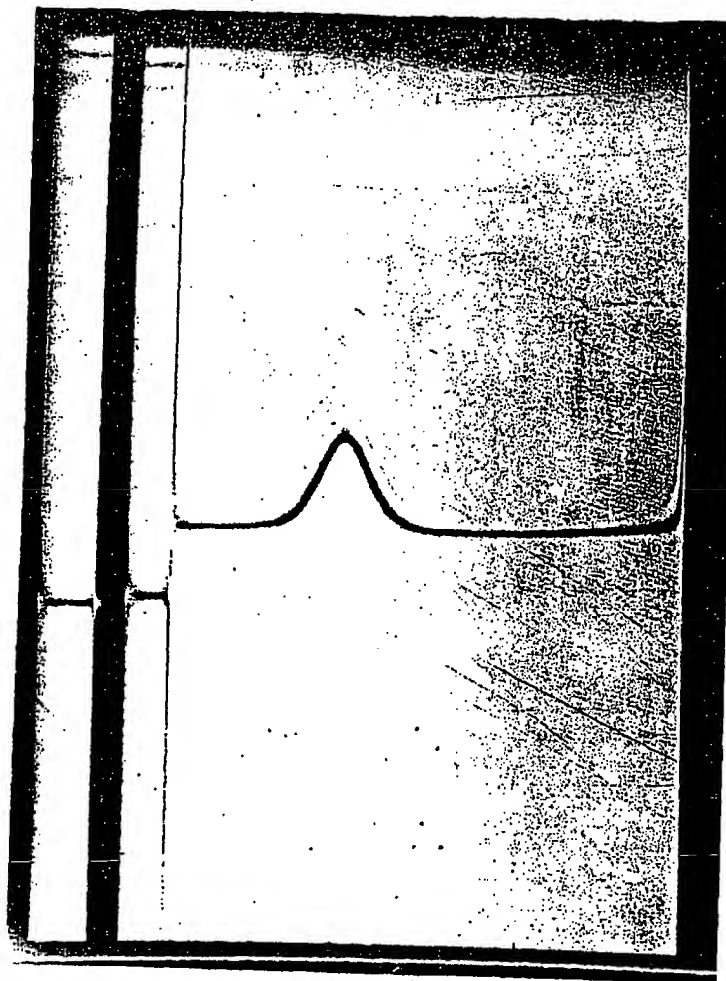


FIG. 4. Sedimentation pattern of purified creatinase. Ultracentrifugal sedimentation run was operated at 60,000 rpm at 20°C with 0.416% enzyme solution in 50 mM acetate buffer containing 0.1 M NaCl, pH 5.8. The photograph was taken 43 min after reaching full speed. Bar angle was 70°.

s^{-1} , respectively, from Lineweaver-Burk plots.

The effects of some chemicals and metal salts on the activity are shown in Table II. The enzyme was completely inhibited by incubation with 0.5 mM PCMB and 1 mM Hg^{2+} , Cu^{2+} , or Zn^{2+} at pH 7.2 for 30 min.

Physicochemical Properties and Subunit Structure

The enzyme showed a maximum absorption at 278 nm and a value of 11.1 was obtained as $A_{1\%}^{1\text{cm}}$ at 280 nm from the measurements of absorbance and dry weight of the purified enzyme.

The molecular weight of the enzyme was estimated by the meniscus depletion method of Yphantis (19). Logarithmic

plots of fringe displacement, $\ln f$, versus the square of radial distance, r^2 , were almost linear (Fig. 5), indicating the homogeneity of the purified enzyme. The molecular weight of the creatinase was estimated to be $94,000 \pm 2000$, assuming that the partial specific volume is $0.73 \text{ (cm}^3/\text{g)}$, which was calculated from the amino acid composition.

Determination of the isoelectric point by the isoelectric focusing method gave a value of 4.7 ± 0.05 (Fig. 6). ORD parameters, a_0 and b_0 , of the purified creatinase were estimated to be -70 and -98 , respectively, suggesting that the α -helix content is less than 20%. The sedimentation coefficient, $S_{20,w}$, of the enzyme was calculated to be 6.3. The sugar content of

the enzyme was lower than 0.14% when calibrated as glucose.

The purified enzyme was subjected to

TABLE II
EFFECTS OF VARIOUS REAGENTS UPON CREATINASE ACTIVITY^a

Chemicals	Concentration (mM)	Inhibition (%)
DFP	1	30
EDTA	2	10
NBS	1	30
PCMB	0.5	100
HgCl ₂	1	100
ZnCl ₂	1	100
CuSO ₄	1	100
CoCl ₂	1	71
MgSO ₄ · 7H ₂ O	1	31
CaCl ₂	1	32
Pb acetate	1	15
FeCl ₂	1	15

^a The enzyme was incubated with the reagents shown above for 30 min at pH 7.2. The remaining activities were assayed by the standard method after 20-fold dilution with 50 mM phosphate buffer, pH 8. DFP, diisopropylfluorophosphate; EDTA, ethylenediaminetetraacetate-2 sodium salt; NBS, *N*-bromosuccinimide.

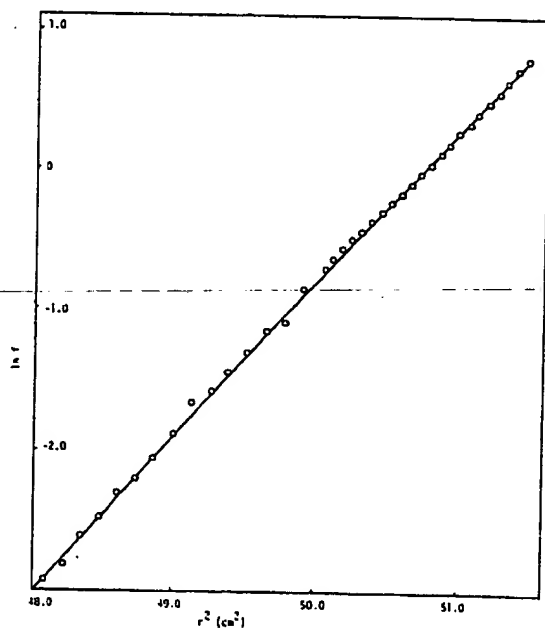


FIG. 5. Sedimentation equilibrium of creatinase by the meniscus depletion method. The experiment was performed at 14,000 rpm with a 12-mm double sector cell containing 0.038% protein solution in 50 mM acetate buffer-0.1 M NaCl, pH 5.8. r , radial distance; f , fringe displacement.

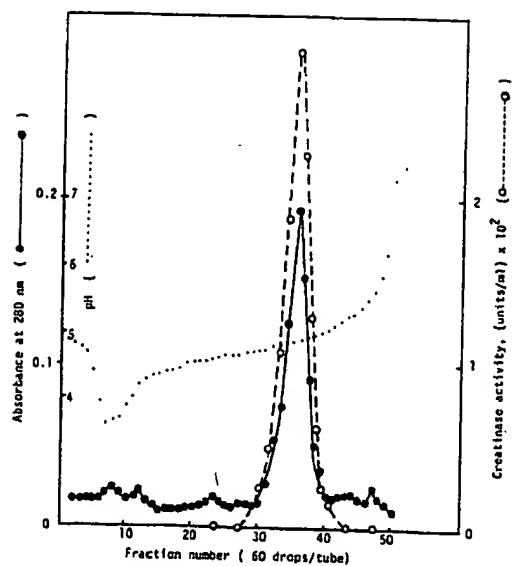


FIG. 6. Isoelectric focusing pattern of purified creatinase. About 4 mg of the enzyme was applied. See the text for the experimental details.

SDS-polyacrylamide gel electrophoresis before and after treatment with 2-mercaptoethanol and a value of 47,000 was obtained as the molecular weight of the enzyme, irrespective of the mercaptoethanol treatment. From the comparison with the value (94,000) estimated by ultracentrifugal method,⁴ it is likely that the creatinase is made up of two subunit monomers noncovalently associated with each other.

Amino Acid Composition and Titration of Sulfhydryl Group

In Table III, the amino acid composition of the creatinase is presented. The number of amino acid residues was calculated assuming that the molecular weight of the monomer is 47,000. The enzyme subunit was found to be composed of 385 residues of amino acids.

As shown in Table II, the enzyme was completely inhibited by incubation with 0.5 mM PCMB. Titration of the creatinase by PCMB following the method of Boyer (20) is shown in Fig. 7. Creatinase activity was completely lost at the inflection point that corresponds to about one sulfhydryl group per mole of subunit.

⁴ Estimation of molecular weight by the gel filtration method of Andrews (21) yielded a value of about 110,000.

Treatment of the enzyme with sodium tetrathionate also resulted in loss of activity, and subsequent treatment with a twofold molar excess of dithiothreitol

TABLE III
AMINO ACID COMPOSITION OF CREATINASE

Amino acid	Number of amino acid residues per mole of subunit ^a	
	Found	Integral
Lysine ^b	12.8	13
Histidine ^b	11.0	11
Arginine ^b	23.5	24
Aspartic acid ^b	45.3	45
Threonine ^c	13.6	14
Serine ^c	11.1	11
Glutamic acid ^b	51.1	51
Proline ^b	15.7	16
Glycine ^b	26.5	27
Alanine ^b	37.0	37
Half cystine ^d	1.7	2
Valine ^e	30.3	30
Methionine ^e	11.7	12
Isoleucine ^e	29.0	29
Leucine ^b	27.8	28
Tyrosine ^b	18.2	18
Phenylalanine ^b	12.0	12
Tryptophan ^f	5.2	5

^a The molecular weight of the subunit was assumed to be 47,000.

^b Average value of 24-, 48-, and 72-h hydrolysates.

^c Value extrapolated to zero hydrolysis time.

^d From the data of performic acid-oxidized preparation.

^e Maximum value was adopted.

^f Spectrophotometrically determined.

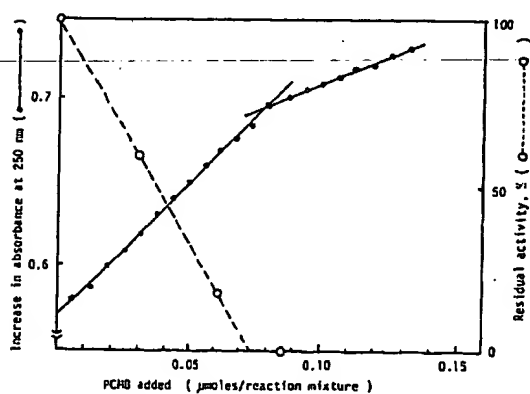


FIG. 7. Titration of free SH group in creatinase with PCMB. The reaction mixtures consisted of 0.0329 μ mol of enzyme and varied concentrations of PCMB in 3 ml of 10 mM Tris-HCl buffer, pH 7.2. After incubation for 30 min at room temperature, the increase in absorbance at 250 nm was measured against the same concentration of PCMB.

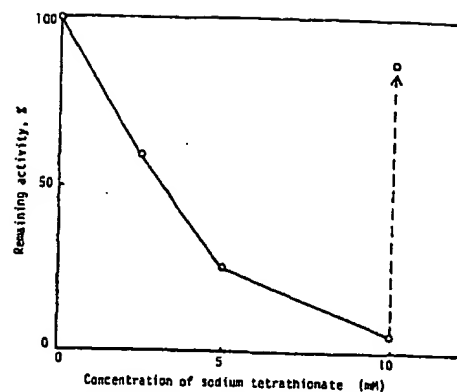


FIG. 8. Inactivation and reactivation of creatinase by treatments with sodium tetrathionate and with dithiothreitol. The reaction mixtures contained 60 μ g of enzyme and varied concentrations of sodium tetrathionate in 1 ml of 50 mM Tris-HCl buffer, pH 7.2, and were incubated at room temperature. The remaining activities were assayed 30 min after incubation. The arrow indicates the restoration of the enzyme activity by treatment with a twofold molar excess of dithiothreitol. Since dithiothreitol was found to inhibit the color development by the α -naphthol-diacetyl method (1) used for creatinase assay, the activity was determined after removal of dithiothreitol by dialysis.

brought about an almost complete restoration of the creatinase activity (Fig. 8). These results suggest that sulfhydryl group plays an important role for the creatinase action.

The present results lead to the conclusion that the creatinase from *P. putida* is made up of two subunit monomers which have molecular weights of 47,000, and that one sulfhydryl group per subunit seems to be closely related to the enzyme action.

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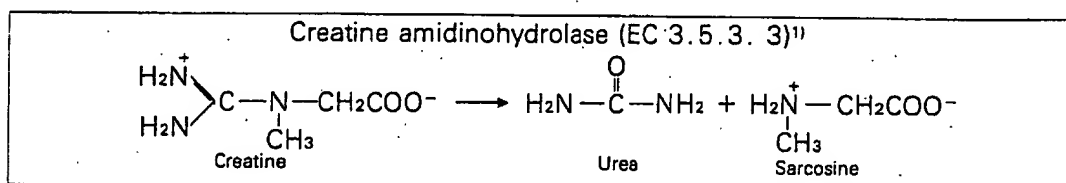
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TOYOBO ENZYMES
(Diagnostic Reagent Grade)

Attachment D

CREATINE AMIDINOHYDROLASE

from *Pseudomonas* sp.



2 PREPARATION and SPECIFICATION

Appearance	: White amorphous powder, lyophilized
Activity	: Grade II 6.0 U/mg-solid or more (containing approx. 40% of stabilizers)
Contaminant	: NADH oxidase $\leq 5.0 \times 10^{-2}\%$ Catalase $\leq 2.0\%$
Stability	: Stable at -20°C for at least 6 months
Stabilizers	: Sucrose, EDTA, 2-mercaptoethanol, dithiothreitol

(Fig. 1)

PROPERTIES^{2, 3)}

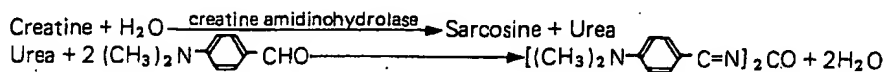
Molecular weight	: approx. 94,000 ²⁾	
Isoelectric point	: 4.70 ± 0.05 ²⁾	
Michaelis constant	: 2.9 × 10 ⁻² M (Creatine)	
Structure	: 2 subunits per mol of enzyme ²⁾	
Inhibitors	: CH ₂ ICOOH, Cu ⁺⁺ , Hg ⁺⁺ , Ag ⁺²)	
Optimum pH	: 7.5	(Fig. 2)
Optimum temperature	: 37°C	(Fig. 3)
pH Stability	: pH 7.5 – 8.0 (5°C, 16 hr)	(Fig. 4)
Thermal stability	: below 37°C (pH 7.5, 30 min.)	(Fig. 5)
Effect of various metals	: (Table 1)	

APPLICATIONS

The enzyme is useful for enzymatic determination of creatine and creatinine by coupling with sarcosine dehydrogenase (or sarcosine oxidase) and formaldehyde dehydrogenase in clinical analysis.⁴⁾

■ ASSAY

Principle:



The appearance of yellow dye formed by condensation of urea and p-dimethylaminobenzaldehyde (DAB) (Ehrlich reaction) is measured at 435nm by spectrophotometry.

Unit definition:

One unit causes the formation of one micromole of yellow dye per minute under the conditions described below.

Method:

Reagents

- A. Creatine solution : 0.1 M [1.49g creatine (Merck)/100ml of 50 mM phosphate buffer, pH7.5] (Prepare freshly)
- B. DAB solution : Dissolve 2.0 g of DAB in 100 ml of dimethylsulfoxide and, to this solution, add 15 ml of conc. HCl solution.
- C. Enzyme diluent : 50 mM phosphate buffer, pH 7.5

Procedure

1. Pipette 0.9 ml of the substrate solution (A) into a test tube and equilibrate at 37°C for about 5 minutes.
2. Add 0.1 ml of the enzyme solution* and mix.
3. After exactly 10 minutes at 37°C, add 2.0 ml of DAB solution (B) to stop the reaction.
4. Incubate at 25°C for 20 minutes.
5. Measure the optical density at 435 nm against water (OD test).

At the same time, prepare the blank by first mixing the substrate solution with 2.0 ml of DAB solution after 10 min-incubation at 37°C, followed by addition of the enzyme solution, and carry out the same procedure as test (procedure 4 and 5) (OD blank).

Concentration in assay mixture	
Phosphate buffer	50 mM
Creatine	90 mM

- * Dissolve the enzyme preparation in ice-cold enzyme diluent (C) and dilute to 2.0–3.0 U/ml with the same buffer, immediately before assay.

Calculation

Activity can be calculated by using the following formula:

$$\begin{aligned} \text{Volume activity (U/ml)} &= \frac{\Delta\text{OD (OD test - OD blank)} \times V_t \times df}{0.321 \times 1.0 \times t \times V_s} \\ &= \Delta\text{OD} \times 9.35 \times df \end{aligned}$$

$$\text{Weight activity (U/mg)} = (\text{U/ml}) \times 1/C$$

- V_t : Total volume (3.0 ml)
- V_s : Sample volume (0.1 ml)
- 0.321 : Millimolar extinction coefficient of yellow dye (cm²/micromole)
- 1.0 : Light path length (cm)
- t : Reaction time (10 minutes)
- df : Dilution factor
- C : Enzyme concentration in dissolution (c mg/ml)

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Table 1. Effect of Various Metals [The enzyme dissolved in 50mM phosphate buffer, pH 7.5 was incubated at 25°C for 30 minutes with various metals (final concn : 0.2 mM)]

Metals	Residual activity	Metals	Residual activity
None	100 %	MgCl ₂	80.3 %
FeSO ₄	91.2	NiCl ₂	1.3
HgCl ₂	0	CoSO ₄	39.9
ZnAc ₂	5.3	BaCl ₂	94.7
CuAc ₂	0	PbAc ₂	83.8
CaAc ₂	61.8	AgNO ₃	0
MnCl ₂	94.7	CdCl ₂	9.2

Ac : CH₃COO-

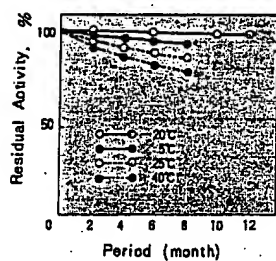


Fig. 1. Stability (Powder form)
[kept under dryness]

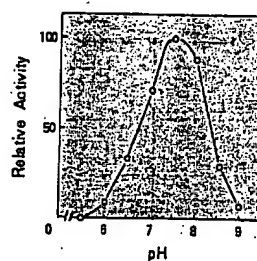


Fig. 2. pH-Activity
[37°C, 10min-reaction in 50mM
buffer solution: pH 5.5, acetate;
pH 6.0-8.0, phosphate; pH 8.5-9.0,
carbonate]

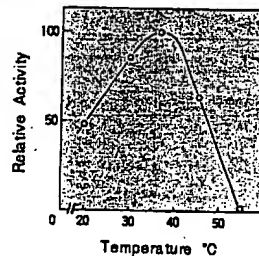


Fig. 3. Temperature activity
[10min-reaction in 50mM
phosphate buffer, pH 7.4]

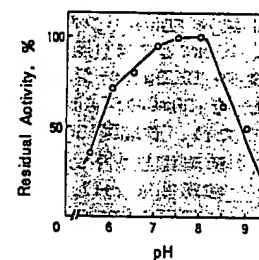


Fig. 4. pH-Stability
[5°C 16hr-treatment with 50mM
buffer solution: pH 5.5,
acetate; pH 6.0-8.0, phosphate;
pH 8.5-9.0, carbonate]

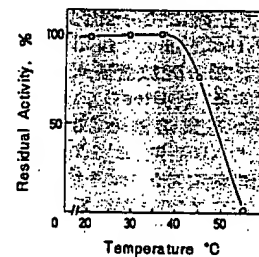
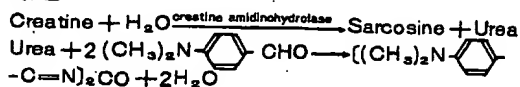


Fig. 5. Thermal stability
[30min-treatment with
50mM phosphate buffer, pH 7.4]

1. 原理



生成した尿素のp-ジメチルアミノベンズアルデヒド(DAB)との縮合 (Ehrlich反応) 生成物 (黄色色素)を比色定量する。

2. 定義

下記条件下で1分間に1マイクロモルの黄色色素を生成する酵素活性を1単位 (U) とする。

3. 試薬

A. 0.1Mクレアチン溶液 (1.49gのクレアチン (Merck製)を50mMリン酸緩衝液 pH7.5に溶解し、100mlとする) (用時調製)

B. DAB溶液 (2.0gのp-ジメチルアミノベンズアルデヒドを100mlのジメチルスルホキシドに溶解させた後、濃塩酸15mlを加える)

酵素溶液: 酵素標品を予め氷冷した50mMリン酸緩衝液、pH7.5で溶解し、分析直前に同緩衝液で2.0~3.0U/mlに希釈する。

4. 手順

1. 試験管に基質溶液 (A) 0.9mlを採り、37℃で約5分間予備加温する。
2. 酵素溶液0.1mlを加え、反応を開始する。
3. 37℃で正確に10分間反応させた後、DAB溶液 (B) 2.0mlを加えて反応を停止させる。
4. 25℃で20分間放置後、435nmにおける吸光度を測定する (ODtest)。
5. 盲検は基質溶液 (A) 0.9mlを37℃で10分間放置後、DAB溶液 (B) 2.0mlを加えて混和し、次いで酵素溶液0.1mlを加えて調製する。以下同様に25℃で20分間放置後吸光度を測定する (ODblank)。

5. 計算式

$$\text{U/ml} = \frac{\Delta \text{OD (ODtest - ODblank)} \times 3.0 \text{ (ml)} \times \text{希釈倍数}}{0.321 \times 1.0 \times 10 \text{ (分)} \times 0.1 \text{ (ml)}}$$

$$= \Delta \text{OD} \times 9.35 \times \text{希釈倍数}$$

$$\text{U/mg} = \text{U/ml} \times 1/\text{C}$$

0.321: 黄色色素のミリモル分子吸光係数 (cm/micromole)

1.0: 光路長 (cm)

C: 溶解時の酵素濃度 (mg/ml)



Creatinase, Pseudomonas species, recombinant

Attachment E

Catalog No.	Lyophilizate, covalently bound to dextran 1035 142
SPECIFICATION	
Appearance	white to slightly yellowish lyophilizate
Solubility	clear solution in water (c = 40 mg/ml)
pH Value	6.5 - 6.9 (40 mg/ml water)
Activity (25°C, creatine, POD/PAP method)	≥ 1.0 U/mg lyophilizate
Activity (25°C, creatine, UV method)	≥ 0.8 U/mg lyophilizate
Storage	dry at 4°C
Stability	≥ 12 months

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ASSAY PROCEDURE

Creatine + H₂O $\xrightarrow{\text{creatinease}}$ sarcosine + urea

sarcosine + O₂ + H₂O $\xrightarrow{\text{sarcosine OD}}$ glycine + HCHO + H₂O₂

H₂O₂ + 4-aminantipyrene + TBHB $\xrightarrow{\text{POD}}$ benzoylironimine dye-stuff + 2 H₂O + HBr

The increase in absorbance is measured at 546 nm.

Reagents:

- Potassium phosphate buffer (0.1 mol/l, pH 7.8):
a) 1.36 g K₂HPO₄/100 ml repurified water;
b) 2.28 g K₂HPO₄ · 3 H₂O or 1.74 g K₂HPO₄/100 ml repurified water; adjust the pH of (b) to 7.8 by adding (a).
- Creatine (0.1 mol/l): 1.31 g creatine, anhydrous/100 ml buffer (1) (prepare freshly every day).
- 4-Aminantipyrene (30 mmol/l): 61 mg 4-aminantipyrene (BM Cat. No. 0073 414)/10 ml repurified water.
- TBHB (143.5 mmol/l, suspended 538 mg 2,4,6-trichloro-3-hydroxybenzoic acid (BM Cat. No. 0755 745)/ ~ 5 ml repurified water; adjust pH to 7.8 with NaOH, 2 mol/l. Adjust volume to 10 ml with repurified water.
- Sarcosine oxidase (150 U/ml) from microorganism, recombinant (BM Cat. No. 1378 858); ~ 30 U/mg lyophilizate; dissolve 10 mg/2 ml repurified water.
- POD (500 U/ml) from horse-mesh (BM Cat. No. 0121 606); ~ 250 U/mg lyophilizate; dissolve 2 mg/1 ml repurified water.

Sample:

volume activity should be ~ 0.08 - 0.22 U/ml.

For lyophilized enzyme, dissolve 10 mg lyophilizate in 1 ml repurified water, dilute 1:250 with buffer (1).

For lyophilized enzyme, covalently bound to dextran: dissolve 10 mg lyophilizate in 1 ml repurified water, dilute 1:50 with buffer (1).

Light path: 1 cm Total volume: 2.82 ml Wavelength: $\epsilon_{546} = 18.0 \times 10^3 \text{ l} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$
Temperature: 25°C Sample volume: 0.05 ml

Pipette into cuvette:

creatine	(2)	2.50 ml	The concentration of the substrate is optimal with ~ 65%. (K_m is too high).
4-aminantipyrene	(3)	0.05 ml	
TBHB	(4)	0.10 ml	
sarcosine OD	(5)	0.10 ml	
POD	(6)	0.02 ml	mix, incubate, check the temperature and determine $\Delta A/\text{min}$ of the pre-reaction. Start the reaction by addition of
sample		0.05 ml	

$\Delta A/\text{min}$ should be ≤ 0.070.
mix, incubate, check the temperature and determine $\Delta A/\text{min}$ of the pre-reaction. Start the reaction by addition of sample. read the change in absorbance after ~ 5 min and calculate $\Delta A/\text{min}$ using the linear portion of the curve (~ 5 min lag phase), 5th reagent disturb.

Calculation:

one unit is the enzyme activity, which catalyzes 1 μmole of creatine per min under the assay conditions (25°C, pH 7.8).

$$\Delta A/\text{min} = \frac{\Delta A/\text{min}_{\text{sample}} - \Delta A/\text{min}_{\text{pre-reaction}}}{\text{sample volume}}$$

$$\text{Volume activity} = \frac{2.82}{18.0 \times 1 \times 0.05} \times \Delta A/\text{min} [\text{U}/\text{ml sample solution}]$$

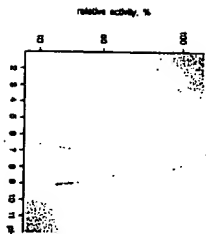
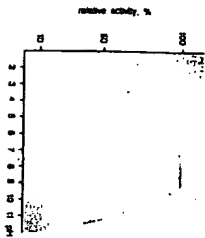
$$\text{Activity}_{50} = \frac{\text{volume activity} \times 250 (50)}{10} [\text{U}/\text{mg lyophilizate}]$$

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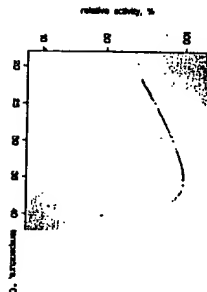
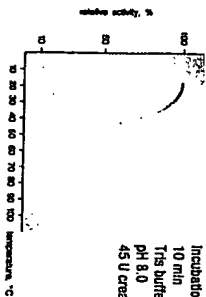
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EXPERIMENTAL DATA

Determination of enzyme activity according to above assay procedure.

Figure 1: pH Optimum
at different pH values.**Figure 3: pH Stability**

Incubation:
25°C, 60 min
pH 3.0 - 5.0:
citrate buffer, 0.1 mol/l
pH 6.0 - 8.0:
phosphate buffer, 0.1 mol/l
pH 9.0 - 11.0:
glycine buffer, 0.1 mol/l
0.9 U creatinase/ml

Figure 2: Temperature Dependence
at different temperatures.**Figure 4: Thermal Stability**

Incubation:
10 min
Tris buffer, 0.1 mol/l,
pH 8.0
45 U creatinase/ml

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 - 6) M. Yasuhara et al., Ciba, Chim. Acta 122, 181-188 (1982)
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PROPERTIES**Origin:**

pseudomonas species creatinase, expressed in recombinant E.coli

Nomenclature:

creatin amidohydrolase, EC 3.5.3.3

Molecular weight:

94 000 (gel filtration)

Structure:

2 subunits of 47 000 (SDS-gel electrophoresis). One SH-group per subunit is necessary for the enzyme reaction.

isoelectric point:

4.8 (isoelectric focusing)

Michaelis constant:phosphate buffer, 10 mmol/l, pH 7.0, 25°C
creatin
 1×10^{-2} mol/l**Inhibitors:** Hg^{2+} , Zn^{2+} , Ag^{+} , Ni^{2+} , Cd^{2+} and Cu^{2+} at a concentration of 1 mmol/l, Fe^{2+} , Pb^{2+} , Ca^{2+} , Co^{2+} and Mg^{2+} at higher concentrations. 4-chloromercuribenzoate, 0.5 mmol/l inhibits completely.**Activators:**

higher activity in Tris buffer as in glycylglycine or TEA.

pH Optimum:

8.0 (fig. 1)

Temperature dependence: see fig. 2**pH Stability:**

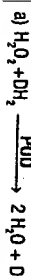
6.0 - 9.0 (fig. 3)

Thermal stability:

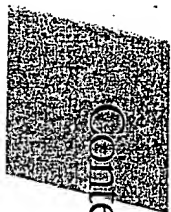
below 30°C (fig. 4)

Specificity:

creatin (100), guanidinoacetate (1), carbanoyl-sarcosine (0), sarcosine (0).

APPLICATIONS AND RELATED PRODUCTS**Creatine:**

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(C12N 1/21
C12R 1:19)
(C12N 9/80
C12R 1:06)
(C12N 9/80
C12R 1:19)

(72)Inventor : TODA ATSUSHI
NISHIYA YOSHIAKI
KAWAMURA YOSHIHISA

PROBLEM TO BE SOLVED: To obtain the subject new enzyme, comprising a creatine amidinohydrolase capable of decomposing creatine into sarcosine and urea in the presence of water, available in a large amount in high purity according to a genetic engineering technique and useful for determination, etc., of creatine and creatinine.

SOLUTION: This creatine amidinohydrolase has actions on creatine in the presence of water and production of sarcosine and urea, about 7.0–8.5 optimum pH, is stable at about $\leq 40^{\circ}\text{C}$ (by treatment at pH7.5 for 30min) and has the pH stability of about 5.0–9.0 (by treatment at 25°C for 16hr), about 46mM value of K_m for the creatine and a molecular weight of about 50,000 [measured by a sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)], 47,146 (a calculated value from the amino acid composition) and about 78,000 (measured by a gel filtration) and further about 4.3 isoelectric point and is represented by the formula. The creatine amidinohydrolase is used for the determination, etc., of the creatine and creatinine. The enzyme is obtained by expressing a gene cloned from a chromosomal DNA of *Arthrobacter* sp. TE1826.

[Date of sending the examiner's decision of

rejection]

[Kind of final disposal of application other than
the examiner's decision of rejection or
application converted registration]

[Date of final disposal for application]

[Patent number]

[Date of registration]

[Number of appeal against examiner's decision
of rejection]

[Date of requesting appeal against examiner's
decision of rejection]

[Date of extinction of right]

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partial translation (JP Publication No. 10-257890)

Claim 1 (page 2, left column, lines 2-15)

Claim 1. A novel creatine amidinohydrolase having the following physicochemical properties:

Action: acting on creatine in the presence of water to produce sarcosine and urea

Optimal temperature: about 40°C

Optimal pH: about 7.0-8.5

Heat stability: about 40°C or below (treated at pH 7.5 for 30 min)

pH stability: about 5.0-9.0 (treated at 25°C for 16 hr)

Km value for creatine: about 46 mM

Molecular weight: about 50,000 (SDS-PAGE)

47,146 (calculated from amino acid composition)

about 78,000 (gel filtration)

Isoelectric point: about 4.3

page 3, left column, lines 2-12

[0005]

[means of solving the problem] The present inventors have investigated variously in an attempt to solve the above object, and selected *Arthrobacter sp.* TE1826 (FERM P-10637) as a creatine amidinohydrolase-producing bacterial strain, and isolated a novel creatine amidinohydrolase from said bacterial strain. Additionally, the present inventors have isolated a recombinant creatine amidinohydrolase expressed from a gene encoding a creatine amidinohydrolase by successively separating said gene from chromosomal DNA extracted from said bacterial strain.

page 3, left column, lines 17-30

[0006] Accordingly, the present invention relates to a novel creatine amidinohydrolase having the following physicochemical properties:

Action: acting on creatine in the presence of water to produce sarcosine

and urea

Optimal temperature: about 40°C

Optimal pH: about 7.0-8.5

Heat stability: about 40°C or below (treated at pH 7.5 for 30 min)

pH stability: about 5.0-9.0 (treated at 25°C for 16 hr)

Km value for creatine: about 46 mM

Molecular weight: about 50,000 (SDS-PAGE)

47,146 (calculated from amino acid composition)

about 78,000 (gel filtration)

Isoelectric point: about 4.3

page 5, right column, lines 25-38

[0030] The creatine amidinohydrolase of the present invention has the following physicochemical properties:

Action: acting on creatine in the presence of water to produce sarcosine and urea

Optimal temperature: about 40°C

Optimal pH: about 7.0-8.5

Heat stability: about 40°C or below (treated at pH 7.5 for 30 min)

pH stability: about 5.0-9.0 (treated at 25°C for 16 hr)

Km value for creatine: about 46 mM

Molecular weight: about 50,000 (SDS-PAGE)

47,146 (calculated from amino acid composition)

about 78,000 (gel filtration)

Isoelectric point: about 4.3

(11)特許出願公開番号

(43)公開日 平成10年(1998)9月29日

審査請求 未請求 請求項の数10 OL (全 13 頁) 最終頁に続く

(54) 【発明の名称】 新規なクレアチンアミジノヒドロラーゼ

【解決手段】 至適温度約40℃、至適pH約7.0～8.5、熱安定性約40℃以下、pH安定性約5.0～9.0であり、かつ、Km値が約46mMであるアースロバクター属細菌由来のクレアチンアミジノヒドロラーゼおよび該酵素をコードする遺伝子、該遺伝子を含有する組換えベクター、該ベクターで形質転換した形質転換体、並びに該形質転換体を培養し、クレアチンアミジノヒドロラーゼを生成させ、該酵素を採取する製造法。

896	1'
897	1'
898	81'
899	77'
900	160'
901	153'
902	240'
903	320'
904	333'
905	313'
906	400'
907	392'

【特許請求の範囲】

【請求項1】 下記理化学的性質を有する新規なクレアチンアミノヒドロラーゼ。

作用：水の存在下にクレアチンに作用して、ザルコシンおよび尿素を生成する。

至適温度：約40℃

至適pH：約7.0～8.5

熱安定性：約40℃以下（pH7.5、30分間処理）

pH安定性：約5.0～9.0（25℃、16時間処理）

クレアチンに対するKm値：約46mM

分子量：約50,000（SDS-PAGE）

47,146（アミノ酸組成から求めた計算値）

約78,000（ゲル濾過）

等電点：約4.3

【請求項2】 以下の（a）又は（b）のタンパク質であるクレアチンアミノヒドロラーゼ。

（a）配列表の配列番号1に記載されるアミノ酸配列からなるタンパク質。

（b）アミノ酸配列（a）において、1もしくは複数のアミノ酸が欠失、置換もしくは付加されたアミノ酸配列からなり、かつ、クレアチンアミノヒドロラーゼ活性を有するタンパク質

【請求項3】 配列表の配列番号1に記載されるアミノ酸配列を有する請求項1記載のクレアチンアミノヒドロラーゼ。

【請求項4】 以下の（a）又は（b）のタンパク質であるクレアチンアミノヒドロラーゼをコードする遺伝子。

（a）配列表の配列番号1に記載されるアミノ酸配列からなるタンパク質。

（b）アミノ酸配列（a）において、1もしくは数個のアミノ酸が欠失、置換もしくは付加されたアミノ酸配列からなり、かつ、クレアチンアミノヒドロラーゼ活性を有するタンパク質

【請求項5】 配列表の配列番号1に記載されるアミノ酸配列からなるタンパク質であるクレアチンアミノヒドロラーゼをコードする遺伝子。

【請求項6】 以下の（c）、（d）または（e）のDNAからなるクレアチンアミノヒドロラーゼをコードする遺伝子。

（c）配列表の配列番号2に記載される塩基配列からDNA

（d）上記（c）の塩基配列において、1もしくは複数の塩基が付加、欠失または置換されており、かつ、クレアチンアミノヒドロラーゼ活性を有するアミノ酸配列をコードしているDNA

（e）上記（c）の塩基配列からなるDNAとストリンジェントな条件下でハイブリダイズし、かつ、クレアチンアミノヒドロラーゼ活性を有するタンパク質をコー

ドする細菌由来のDNA

【請求項7】 請求項4、5または6記載のクレアチンアミノヒドロラーゼをコードする遺伝子を含有する組換えベクター。

【請求項8】 請求項7記載の組換えベクターで宿主細胞を形質転換した形質転換体。

【請求項9】 請求項8記載の形質転換体を培養し、クレアチンアミノヒドロラーゼを生成させ、該クレアチンアミノヒドロラーゼを採取することを特徴とするクレアチンアミノヒドロラーゼの製造法。

【請求項10】 アースロバクター・エスピー（*Arthrobacter* sp.）TE1826（FERM P-10637）を培養し、クレアチンアミノヒドロラーゼを生成させ、該クレアチンアミノヒドロラーゼを採取することを特徴とするクレアチンアミノヒドロラーゼの製造法。

【発明の詳細な説明】

【0001】

【発明の属する技術分野】本発明はクレアチンおよびクレアチニンの定量に用いることのできるクレアチンアミノヒドロラーゼ活性を有する新規な酵素、ならびに該酵素をコードする遺伝子および遺伝子工学的技術による該酵素の製造法に関する。

【0002】

【従来の技術】従来から、クレアチンアミノヒドロラーゼ（EC 3.5.3.3）は、臨床的に筋疾患、腎疾患の診断の指標となっている体液中のクレアチンおよびクレアチニンの測定用酵素として、他の酵素、例えばクレアチンアミドヒドロラーゼ、ザルコシンオキシダーゼおよびベルオキシダーゼと共に使用されている。クレアチンアミノヒドロラーゼは、水の存在下にクレアチンに作用して、ザルコシンと尿素を生成する反応を触媒する酵素である。

【0003】このようなクレアチンアミノヒドロラーゼは、シュードモナス属（*Journal of Biochemistry*, Vol. 79, 1381-1383 (1976)）あるいはバチルス属（特公昭61-17465）等の細菌が生産することが知られている。さらに、これら以外の細菌としては、フラボバクテリウム属、コリネバクテリウム属、マイクロコッカス属（特開昭51-11884号公報）、アルカリゲネス属、ペニシリウム属（特開昭47-43281号公報）などの細菌が知られているに過ぎない。

【0004】

【発明が解決しようとする課題】本発明の目的は、今までに生産していない新しい細菌から、新規なクレアチンアミノヒドロラーゼを単離し、そして、該酵素をコードする遺伝子をクローニングし、遺伝子工学的に該酵素を多量に製造する方法を提供することにある。また、本発明の別な目的は、該遺伝子を改変することにより、理化学的性質の改良された新規なクレアチンアミノヒド

ローラーゼを製造する方法を導き出すことにある。

【0005】

【課題を解決するための手段】本発明者らは、上記目的を達成するために種々検討した結果、クレアチンアミジノヒドロラーゼ生産菌として、アースロバクター・エスピー(Arthrobacter sp.)TE1826(FERM P-10637)を選び、該菌株から新規なクレアチンアミジノヒドロラーゼを単離し、さらに、該菌体より抽出した染色体DNAからクレアチンアミジノヒドロラーゼをコードする遺伝子を分離することに成功し、該遺伝子より発現される、組換えクレアチンアミジノヒドロラーゼを単離した。さらに、該遺伝子の全塩基配列を決定し、該酵素を遺伝子工学的技術により高価なクレアチニンのような誘導物質を培地に添加する必要なく、高生産させることに成功し、高純度な該酵素を安価に大量供給することを可能にした。

【0006】すなわち、本発明は下記理化学的性質を有する新規なクレアチンアミジノヒドロラーゼである。
作用：水の存在下にクレアチンに作用して、ザルコシンおよび尿素を生成する。
至適温度：約40℃
至適pH：約7.0～8.5
熱安定性：約40℃以下(pH7.5、30分間処理)
pH安定性：約5.0～9.0(25℃、16時間処理)
クレアチンに対するKm値：約46mM
分子量：約50,000(SDS-PAGE)
47,146(アミノ酸組成から求めた計算値)
約78,000(ゲル濾過)
等電点：約4.3

【0007】また、本発明は以下の(a)又は(b)のタンパク質であるクレアチンアミジノヒドロラーゼである。

(a)配列表の配列番号1に記載されるアミノ酸配列からなるタンパク質。

(b)アミノ酸配列(a)において1もしくは複数のアミノ酸が欠失、置換もしくは付加されたアミノ酸配列からなり、かつ、クレアチンアミジノヒドロラーゼ活性を有するタンパク質

【0008】本発明は、以下の(a)又は(b)の組換えタンパク質であるクレアチンアミジノヒドロラーゼをコードする遺伝子である。

(a)配列表の配列番号1に記載されるアミノ酸配列からなるタンパク質。

(b)アミノ酸配列(a)において、1もしくは複数のアミノ酸が欠失、置換もしくは付加されたアミノ酸配列からなり、かつ、クレアチンアミジノヒドロラーゼ活性を有するタンパク質

【0009】また、本発明は以下の(c)、(d)または(e)のDNAからなるクレアチンアミジノヒドロ

ラーゼをコードする遺伝子である。

(c)配列表の配列番号2に記載される塩基配列からなるDNA

(d)上記(c)の塩基配列において、1もしくは複数の塩基が付加、欠失または置換されており、かつ、クレアチンアミジノヒドロラーゼ活性を有するアミノ酸配列をコードしているDNA

(e)上記(c)の塩基配列からなるDNAとストリンジントな条件下でハイブリダイズし、かつ、クレアチンアミジノヒドロラーゼ活性を有するタンパク質をコードする細菌由来のDNA

【0010】本発明は上記クレアチンアミジノヒドロラーゼをコードする遺伝子を含有する組換えベクターである。

【0011】また、本発明は上記組換えベクターで宿主細胞を形質転換した形質転換体である。

【0012】さらに、本発明は上記形質転換体を培養し、クレアチンアミジノヒドロラーゼを生成させ、該クレアチンアミジノヒドロラーゼを採取することを特徴とするクレアチンアミジノヒドロラーゼの製造法である。

【0013】また、本発明はアースロバクター・エスピー(Arthrobacter sp.)TE1826(FERM P-10637)を培養し、クレアチンアミジノヒドロラーゼを生成させ、該クレアチンアミジノヒドロラーゼを採取することを特徴とするクレアチンアミジノヒドロラーゼの製造法である。

【0014】

【発明の実施態様】本発明のクレアチンアミジノヒドロラーゼは、クレアチンアミジノヒドロラーゼ生産微生物、例えばアースロバクター・エスピー(Arthrobacter sp.)TE1826(FERM P-10637)から入手し得る。しかしながら、クレアチンアミジノヒドロラーゼをコードする遺伝子を分離し、これより発現されるクレアチンアミジノヒドロラーゼを単離することにより、本発明のクレアチンアミジノヒドロラーゼを簡便、かつ効率的に入手することもできる。本発明の一実施態様としては、(a)配列表の配列番号1に記載されるアミノ酸配列からなるタンパク質または(b)アミノ酸配列(a)において、1もしくは複数のアミノ酸が欠失、置換もしくは付加されたアミノ酸配列からなり、かつ、クレアチンアミジノヒドロラーゼ活性を有するタンパク質がある。アミノ酸の欠失、置換、付加の程度については、基本的な特性を変化させることなく、あるいはその特性を改善するようにしたものを含む。これらの変異体を製造する方法は、従来から公知である方法に従う。

【0015】本発明のクレアチンアミジノヒドロラーゼをコードする遺伝子は、例えばアースロバクター・エスピー(Arthrobacter sp.)TE1826(FERM P-10637)から抽出しても良く、また化学的に合成することもできる。ポリメラーゼチェーンリアクション法

(PCR)の利用により、クレアチンアミジノヒドロラーゼ遺伝子を含むDNA断片を得ることも可能である。

【0016】上記遺伝子としては、例えば、(a)配列表の配列番号1に記載されるアミノ酸配列からなるタンパク質をコードするDNA、または(b)アミノ酸配列(a)において、1もしくは数個のアミノ酸が欠失、置換もしくは付加されたアミノ酸配列からなり、かつ、クレアチンアミジノヒドロラーゼ活性を有するタンパク質をコードするDNAがある。DNAの欠失、置換、付加の程度については、基本的な特性を変化させることなく、あるいはその特性を改善するようにしたものを含む。これらの変異体を製造する方法は、従来から公知である方法に従う。

【0017】または、(c)配列表の配列番号2に記載される塩基配列からDNA、(d)(c)の塩基配列において1もしくは数個の塩基が付加、欠失または置換されており、かつクレアチンアミジノヒドロラーゼ活性を有するアミノ酸配列をコードしているDNAまたは(e)(c)の塩基配列からなるDNAとストリンジェントな条件下でハイブリダイズし、かつ、クレアチンアミジノヒドロラーゼ活性を有するタンパク質をコードする細菌由来のDNAがある。ここで、ストリンジェントな条件とは×2SSC(300mM NaCl、30mM クエン酸)、65℃、16時間である。

【0018】本発明の遺伝子を得る方法としては、例えば、アースロバクター・エスピー(*Arthrobacter* sp.)TE1826(FERM P-10637)の染色体DNAを分離、精製した後、超音波破碎、制限酵素処理等を用いてDNAを切断したものと、リニヤーな発現ベクターとを両DNAの平滑末端または接着末端においてDNAリガーゼなどにより結合閉鎖させて組換えベクターを構築する。こうして得られた組換えベクターは複製可能な宿主微生物に移入した後、ベクターのマーカーと酵素活性の発現を指標としてスクリーニングして、クレアチンアミジノヒドロラーゼをコードする遺伝子を含有する組換えベクターを保持する微生物を得る。次いで該微生物を培養し、該培養菌体から該組換えベクターを分離・精製し、該組換えベクターからクレアチンアミジノヒドロラーゼ遺伝子を採取すれば良い。

【0019】遺伝子供与体であるアースロバクター・エスピー(*Arthrobacter* sp.)TE1826(FERM P-10637)の染色体DNAは、具体的には、以下のように採取される。すなわち、供与微生物を例えば、1～3日間攪拌培養して得られた培養物を遠心分離にて集菌し、次いでこれを溶菌させることによりクレアチンアミジノヒドロラーゼ遺伝子の含有溶菌物を調製することができる。溶菌の方法としては、例えば、リゾチームやβ-グルカナーゼ等の溶菌酵素により処理が施され、必要に応じてプロテアーゼや他の酵素やラウリル硫酸ナトリウム(SDS)等の界面活性剤が併用され、さらに凍結

融解やフレンチプレス処理のような物理的破碎方法と組み合わせても良い。

【0020】このようにして得られた溶菌物からDNAを分離・精製するには、常法に従って、例えばフェノール処理やプロテアーゼ処理による除蛋白処理や、リボヌクレアーゼ処理、アルコール沈澱処理などの方法を適宜組み合わせることにより行うことができる。微生物から分離・精製されたDNAを切断する方法は、例えば、超音波処理、制限酵素処理などにより行うことができる。好ましくは、特定のヌクレオチド配列に作用するII型制限酵素が適している。

【0021】ベクターとしては、宿主微生物内で自律的に増殖し得るファージまたはプラスミドから遺伝子組換え用として構築されたものが適している。ファージとしては、例えば、エシェリヒア・コリー(*Escherichia coli*)を宿主微生物とする場合には、Lambda- ϕ 10、Lambda- ϕ 11などが使用できる。またプラスミドとしては、例えばエシェリヒア・コリー(*Escherichia coli*)を宿主微生物とする場合には、pBR322、pUC19、pBluescript、pLED-M1(Journal of Fermentation and Bioengineering, Vol.76, 265-269 (1993))などが使用できる。このようなベクターを、上述したクレアチンアミジノヒドロラーゼ遺伝子供与体である微生物DNAの切断に使用した制限酵素で切断してベクター断片を得ることができるが、必ずしも該微生物DNAの切断に使用した制限酵素と同一の制限酵素を用いる必要はない。微生物DNA断片とベクターDNA断片とを結合させる方法は、公知のDNAリガーゼを用いる方法であれば良く、例えば微生物DNA断片の接着末端とベクター断片の接着末端とのアニーリングの後、適当なDNAリガーゼの使用により微生物DNA断片とベクターDNA断片との組換えベクターを作成する。必要なら、アニーリングの後、宿主微生物に移入して生体内のDNAリガーゼを利用し組換えベクターを作成することもできる。

【0022】宿主微生物としては、組換えベクターが安定、かつ自律増殖可能で外来性遺伝子の形質発現できるものであれば良く、一般的にはエシェリヒア・コリーW3110、エシェリヒア・コリーC600、エシェリヒア・コリーHB101、エシェリヒア・コリーJM109などを用いることができる。

【0023】宿主微生物に組換えベクターを移入する方法としては、例えば宿主微生物がエシェリヒア・コリーの場合には、カルシウム処理によるコンピテントセル法やエレクトロポレーション法などが用いることができる。

【0024】このようにして得られた形質転換体である微生物は、栄養培地で培養されることにより、多量のクレアチンアミジノヒドロラーゼを安定に生産し得る。宿主微生物への目的組換えベクターの移入の有無についての選択は、目的とするDNAを保持するベクターの薬剤耐性マーカーとクレアチンアミジノヒドロラーゼ活性を

同時に発現する微生物を検索すれば良く、例えば薬剤耐性マーカーに基づく選択培地で生育し、かつ、クレアチンアミジノヒドロラーゼを生成する微生物を選択すれば良い。

【0025】上記の方法により得られたクレアチンアミジノヒドロラーゼ遺伝子の塩基配列はサイエンス (Science, Vol.214, 1205-1210 (1981)) に記載されたジデオキシ法により解読し、またクレアチンアミジノヒドロラーゼのアミノ酸配列は決定した塩基配列より推定した。このようにして一度選択されたクレアチンアミジノヒドロラーゼ遺伝子を保有する組換えベクターは、形質転換微生物から取り出され、他の微生物に移入することも容易に実施することができる。また、クレアチンアミジノヒドロラーゼ遺伝子を保持する組換えベクターから制限酵素やPCRによりクレアチンアミジノヒドロラーゼ遺伝子であるDNAを回収し、他のベクター断片と結合させ、宿主微生物に移入することも容易に実施できる。

【0026】形質転換体である宿主微生物の培養形態は、宿主の栄養生理的性質を考慮して培養条件を選択すればよく、通常、多くの場合は液体培養で行うが、工業的には通気攪拌培養を行うのが有利である。培地の栄養源としては、微生物の培養に通常用いられるものが広く使用され得る。炭素源としては、資化可能な炭素化合物であればよく、例えばグルコース、シュークロース、ラクトース、マルトース、フラクトース、糖蜜、ビルビン酸などが使用される。窒素源としては、利用可能な窒素化合物であればよく、例えばペプトン、肉エキス、酵母エキス、カゼイン加水分解物、大豆粕アルカリ抽出物などが使用される。その他、リン酸塩、炭酸塩、硫酸塩、マグネシウム、カルシウム、カリウム、鉄、マンガン、亜鉛などの塩類、特定のアミノ酸、特定のビタミンなどが必要に応じて使用される。培養温度は菌が発育し、クレアチンアミジノヒドロラーゼを生産する範囲で適宜変更し得るが、エシェリヒア コリーの場合、好ましくは20~42℃程度である。培養時間は条件によって多少異なるが、クレアチンアミジノヒドロラーゼが最高収量に達する時期を見計らって適当時期に培養を終了すればよく、通常は6~48時間程度である。培地pHは菌が発育し、クレアチンアミジノヒドロラーゼを生産する範囲で、適宜変更し得るが、特に好ましくはpH6.0~9.0程度である。

【0027】培養物中のクレアチンアミジノヒドロラーゼを生産する菌体を含む培養液をそのまま採取し、利用することもできるが、一般には、常法に従ってクレアチンアミジノヒドロラーゼが培養液中に存在する場合は、濾過、遠心分離などにより、クレアチンアミジノヒドロラーゼ含有溶液と微生物菌体とを分離した後に利用される。クレアチンアミジノヒドロラーゼが菌体内に存在する場合には、得られた培養物から濾過または遠心分離な

どの手段により菌体を採取し、次いでこの菌体を機械的方法またはリゾチームなどの酵素的方法で破壊し、また必要に応じてEDTA等のキレート剤及びまたは界面活性剤を添加してクレアチンアミジノヒドロラーゼを可溶化し、水溶液として分離採取する。

【0028】このようにして得られたクレアチンアミジノヒドロラーゼ含有溶液を、例えば減圧濃縮、膜濃縮、更に硫酸アンモニウム、硫酸ナトリウムなどの塩析処理、或いは親水性有機溶媒、例えばメタノール、エタノール、アセトンなどによる分別沈澱法により沈澱せしめればよい。また、加熱処理や等電点処理も有効な精製手段である。その後、吸着剤或いはゲル濾過剤などによるゲル濾過、吸着クロマトグラフィー、イオン交換クロマトグラフィー、アフィニティークロマトグラフィーを行うことにより、精製されたクレアチンアミジノヒドロラーゼを得ることができる。

【0029】例えば、セファデックス (Sephadex) G-25 (ファルマシア バイオテック) などによるゲルろ過、DEAEセファロースCL-6B (ファルマシア バイオテック)、オクチルセファロースCL-6B (ファルマシア バイオテック) カラムクロマトグラフィーにより分離・精製し精製酵素標品を得ることができる。この精製酵素標品は、電気泳動 (SDS-PAGE) 的に、ほぼ単一のバンドを示す程度に純化されている。

【0030】本発明のクレアチンアミジノヒドロラーゼは、以下に示す理化学的性質を有する。

作用：水の存在下にクレアチンに作用して、ザルコシンおよび尿素を生成する。

至適温度：約40℃

至適pH：約7.0~8.5

熱安定性：約40℃以下 (pH7.5:30分間処理)

pH安定性：約5.0~9.0 (25℃、16時間処理)

クレアチンに対するKm値：約46mM

分子量：約50,000 (SDS-PAGE)

47,146 (アミノ酸組成から求めた計算値)

約78,000 (ゲル濾過)

等電点：約4.3

【0031】本発明のクレアチンアミジノヒドロラーゼと公知のクレアチンアミジノヒドロラーゼとの性質の比較を表1に示す。また、本発明のクレアチンアミジノヒドロラーゼのアミノ酸配列と公知であるシュドモナス・ブチダ (Pseudomonas putida) が産生するクレアチンアミジノヒドロラーゼのアミノ酸配列との相同性は、63.4%であり、両者の比較を図1に示す。本発明のクレアチンアミジノヒドロラーゼは、同一反応を触媒する公知の酵素とは性質の異なる新規な酵素である。

【0032】

【表1】

由来	Arthrobacter sp. TB1826	Pseudomonas putida var. naraensis C-83	Bacillus sp. B-0618
至適温度	40℃	—	40℃
至適pH	7.0~8.5	8.0	7.5~9.0
熱安定性	40℃以下 (pH7.5, 30分間)	45℃以下 (pH7.4, 30分間)	40℃以下 (pH7.5, 10分間)
pH安定性	5.0~9.0	6.0~8.0	6.0~9.0
クレアチン に対するKm	46	—	—
分子量	50,000 (SDS-PAGE)	47,000 (SDS-PAGE)	—
サブ ユニット	2	2	—
等電点	4.3	4.7	4.9

【0033】

【実施例】以下、本発明を実施例により具体的に説明する。実施例中、クレアチンアミノヒドロラーゼ活性の測定は以下の試薬および測定条件で行った。

<試薬>

試薬混液組成

- 0.3M HEPES pH7.6
- 1.8% クレアチン
- 0.015% フェノール
- 0.005% 4-アミノアンチピリン
- 6U/ml ザルコシンオキシダーゼ
- 6U/ml ベルオキシダーゼ

【0034】<測定条件>上記試薬混液3mlを37℃で約3分予備加温後、0.1mlの酵素溶液を加え、37℃で反応を開始し、4分間反応させた後、500nm 30における1分間当たりの吸光度変化を分光光度計にて測定する。盲検は、酵素溶液の代わりに蒸留水を試薬混液に加えて、以下、同様に吸光度変化を測定する。上記条件で1分間に1マイクロモルのザルコシンを生成する酵素量を1単位(U)とする。

【0035】実施例1 染色体DNAの分離

アースロバクター・エスピーTE1826 (FERM P-10637) の染色体DNAを次の方法で分離した。同菌株を100mlの2×YT培地 (1.0%ポリペプトン、1%酵母エキス、0.5%塩化ナトリウム(pH7.2))で37℃で、一晚振盪培養した後、遠心分離 (8,000rpm, 10分間) により集菌した。15mMクエン酸ナトリウム、0.15M塩化ナトリウムを含んだ溶液で菌体を洗浄した後、20%シュクロース、50mMトリス塩酸 (pH7.6)、1mM EDTAを含んだ溶液5mlに懸濁し、1mlのリゾチーム溶液 (100mq/ml) を加えて、37℃、30分間保温し、次いで11mlの1%ラウロイルサルコシン酸、0.1MEDTA (pH9.6) を含む溶液を加えた。この懸濁液に臭化エチジウム溶液を0.5%塩化セシウムを約100%加え、攪拌混合し、

55,000rpm、20時間の超遠心分離でDNAを分取した。分取したDNAは1mM EDTAを含んだ10mMトリス塩酸、pH8.0溶液 (以下、TEと略記) で透析し、精製DNA標品とした。これを等量のクロロホルム・フェノール溶液で処理後遠心分離により水層を分取し、2倍量のエタノールを加えて上記方法で、もう一度DNAを分離し、2mlのTEで溶解した。

【0036】実施例2 クレアチンアミノヒドロラーゼをコードする遺伝子を含有するDNA断片及び該DNA断片を有する組換えベクターの調製

実施例1で得たDNA5μgを制限酵素 Sau3AI (東洋紡製) で部分分解し、2kbp以上の断片に分解した後、制限酵素 BamHI (東洋紡製) で切断した pBluescriptKS(+) 1μgとをT4-DNAリガーゼ (東洋紡製) 1単位で、16℃、12時間反応させ、DNAを連結した。連結したDNAはエシェリヒア・コリ JM109 のコンピテントセル (東洋紡製) を用いて形質転換し、クレアチンアミノヒドロラーゼ活性検出用寒天培地 [1.0%ポリペプトン、0.5%酵母エキス、0.5% NaCl、1%クレアチン、10U/mlザルコシンオキシダーゼ (東洋紡製)、5U/mlベルオキシダーゼ (東洋紡製)、0.01% o-ジアニシジン、50μg/mlアンピシリン、1.5%寒天] に塗布した。クレアチンアミノヒドロラーゼ活性の検出は、上記培地に生育し、かつ茶色に染色されるコロニーを指標 40 に行った。

【0037】使用したDNA1μg当たり約100,000個の形質転換体のコロニーが得られ、上記スクリーニングの結果、茶色に染色されるコロニーを1株を見いだした。この株をLB液体培地 (1%ポリペプトン、0.5%酵母エキス、0.5% NaCl、50μg/mlアンピシリン) で培養し、クレアチンアミノヒドロラーゼ活性を測定したところ、該活性が検出された。この株の保有するプラスミドには約6.6kbpの挿入DNA断片が存在しており、このプラスミドをpCNAB1とした。

【0038】次いでpCNAB1の挿入DNAを制限酵

素 ApaI (東洋紡製) にて切り出し、同制限酵素で切断した pBluescriptKS(+) に連結して、pCNHAA-1 を作成した。この pCNHAA-1 の制限酵素地図を図2に示す。

【0039】実施例3 塩基配列の決定

pCNHAA-1 の約4.6 kbp の挿入DNA断片について種々の制限酵素にてサブクローンを調製した。種々のサブクローンは常法に従い、Radioactive Sequencing Kit (東洋紡製) を用いて、塩基配列を決定した。決定した塩基配列およびアミノ酸配列を配列表に示した。*10

<反応液組成>

Pfu DNA ポリメラーゼ (ストラタジーン製)	5U/100 μ l
10倍濃度 Pfu DNA ポリメラーゼ用バッファー	10 μ l/100 μ l
pCNHAA-1 (鋳型DNA)	0.1 μ g/100 μ l
dATP, dTTP, dGTP, dCTP	各0.2mM
2種のプライマー	各1 μ M

(配列表の配列番号3, 4に記載)

【0041】<増幅条件>

変性	95℃、30秒間
温度変更	1分30秒間
アニーリング	45℃、1秒間
温度変更	1秒間
反応	75℃、2分30秒間
温度変更	1秒間

(上記サイクルを合計30サイクル実施)

【0042】増幅DNA断片約1.2 kbp を、制限酵素 EcoRI で切断した pBluescript SK(-) に連結して pCRHAR-1 を作成した。pCRHAR-1 の制限酵素地図を図3に示す。pCRHAR-1 の挿入DNA断片は、クレアチンアミノヒドロラーゼ遺伝子以外の部分を含まない。

【0043】実施例5 形質転換体の作成

エシェリヒア・コリー JM109 のコンピテントセル (東洋紡製) を pCRHAR-1 で形質転換し、形質転換体エシェリヒア・コリー JM109(pCRHAR-1) を得た。

【0044】実施例6 エシェリヒア・コリー JM109(pCRHAR-1) からのクレアチンアミノヒドロラーゼの製造 LB培地500mlを2Lフラスコに分注し、121℃、15分間オートクレーブを行い、放冷後、別途、無菌ろ過した50mg/mlアンピシリン (ナカライテスク製) 0.5mlを添加した。この培地にLB培地であらかじめ、30℃、7時間振盪培養したエシェリヒア・コリー JM109(pCRHAR-1) の培養液5mlを接種し、37℃で18時間通気攪拌培養した。培養終了時のクレアチンアミノヒドロラーゼ活性は約5U/mlであった。

【0045】上記菌体を遠心分離にて集菌し、20mMリン酸緩衝液、pH7.5に懸濁した。上記菌体懸濁液をフレンチプレスで破碎し、遠心分離を行い上清液を得た。得られた粗酵素液をポリエチレンイミンによる除核

*アミノ酸配列から求められる蛋白質の分子量は47,146であり、アースロバクター・エスピーTE1826のクレアチンアミノヒドロラーゼの分子量とほぼ一致した。

【0040】実施例4 組換えベクターpCRHAR-1の作成

pCNHAA-1の挿入DNA断片よりクレアチンアミノヒドロラーゼ遺伝子以外の部分を除くため、PCRによる挿入DNA断片の小型化を実施した。PCRは以下に示す反応液組成及び増幅条件にて行った。

(Sephadex) G-25 (ファルマシア バイオテック) によるゲルろ過により脱塩し、DEAEセファロースCL-6B (ファルマシア バイオテック) カラムクロマトグラフィー、オクチルセファロースCL-6B (ファルマシア バイオテック) カラムクロマトグラフィーにより分離・精製し、精製酵素表品を得た。該方法により得られたクレアチンアミノヒドロラーゼ標品は、電気泳動(SDS-PAGE)的にほぼ単一なバンドを示し、この時の比活性は約21U/mgタンパク質であった。

【0046】以下に、上記方法により得られたクレアチンアミノヒドロラーゼの性質を示す。

作用：クレアチンに水の存在下に作用して、ザルコシンおよび尿素を生成する。

至適温度：約40℃

至適pH：約7.0~8.5

熱安定性：約40℃以下 (pH7.5、30分間処理)

pH安定性：約5.0~9.0 (25℃、16時間処理)

クレアチンに対するKm値：約46mM

分子量：約50,000 (SDS-PAGE)

47,146 (アミノ酸組成から求めた計算値)

約78,000 (ゲル濾過)

等電点：約4.3

【0047】実施例7

アースロバクター・エスピー (Arthrobacter sp.) TE1826 (FERM P-10637) を2XYT培地 (1.6%ポリペプトン、1%酵母エキス、0.5%塩化ナトリウム、pH7.2) にて、37℃、約1~3日間、培養し、培養液を遠心分離して集菌し、次いでこれを溶菌させることによって、クレアチンアミノヒドロラーゼを生成させ、該クレアチンアミノヒドロラーゼを採取した。

【0048】

【発明の効果】本発明により、アースロバクター属細菌

から新規なクレアチンアミジノヒドロラーゼ遺伝子が単離され、遺伝子工学的技術による該酵素の製造法が確立され、高純度な該酵素の大量供給とクレアチニンの定量への利用が可能となった。また、該遺伝子をを改変することにより、理化学的性質の改良された新規なクレアチンアミジノヒドロラーゼを製造する方法を導き出すことができる。

【0049】

【配列表】

* 配列番号: 1

配列の長さ: 411

配列の型: アミノ酸

トポロジー: 直鎖状

配列の種類: 蛋白質

起源

生物名: アースロバクター・エスピー (Arthrobacter sp.)

* 株名: TE1826 (FERM P-10637)

配列

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Met Gln Lys Ile Thr Asp Leu Glu Arg Thr Lys Val Leu His Asn Gly
  1           5           10           15
Glu Lys Phe Lys Gly Thr Phe Ser Lys Ala Glu Met Asp Arg Arg Asn
          20           25           30
Thr Asn Leu Arg Asn Tyr Met Ala Glu Lys Asp Ile Asp Ala Val Leu
          35           40           45
Phe Thr Ser Tyr His Asn Ile Asn Tyr Tyr Ser Asp Phe Leu Tyr Thr
          50           55           60
Ser Phe Asn Arg Asn Tyr Gly Leu Val Val Thr Gln Asn Lys His Val
          65           70           75           80
Thr Val Ser Ala Asn Ile Asp Gly Gly Met Pro Trp Arg Arg Ser Tyr
          85           90           95
Asp Glu Asn Ile Val Tyr Thr Asp Trp Arg Arg Asp Asn Tyr Phe Tyr
          100          105          110
Ala Ile Gln Lys Val Leu Glu Glu Ala Gly Val Lys Lys Ala Arg Leu
          115          120          125
Gly Ile Glu Glu Asp His Val Ser Ile Asp Leu Leu Arg Lys Phe Ser
          130          135          140
Asp Thr Phe Pro Asn Phe Glu Leu Val His Val Ser Gln Asp Val Met
          145          150          155          160
Lys Gln Arg Met Ile Lys Ser Ala Glu Glu Ile Arg His Ile Lys Asn
          165          170          175
Gly Ala Arg Ile Ala Asp Ile Gly Gly Tyr Ala Val Val Glu Ala Ile
          180          185          190
Gln Glu Gly Val Pro Glu Tyr Glu Val Ala Leu Ala Gly Ser Lys Ala
          195          200          205
Met Thr Arg Glu Ile Ala Lys Leu Tyr Pro Gln Ser Glu Leu Arg Asp
          210          215          220
Thr Trp Val Trp Phe Gln Ala Gly Ile Asn Thr Asp Gly Ala His Ser
          225          230          235          240
Trp Ala Thr Ser Lys Lys Val Gln Lys Gly Glu Ile Leu Ser Leu Asn
          245          250          255
Thr Phe Pro Met Ile Ala Gly Tyr Tyr Thr Ala Leu Glu Arg Thr Leu
          260          265          270
Phe Leu Glu Glu Val Ser Asp Ala His Leu Lys Tyr Trp Glu Ile Asn
          275          280          285
Val Glu Val His Lys Arg Gly Leu Glu Leu Ile Lys Pro Gly Ala Val
          290          295          300
Cys Lys Asp Ile Cys Ala Glu Leu Asn Glu Met Phe Arg Glu His Asp
          305          310          315          320

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15
Leu Val Lys Asn Arg Thr Phe Gly Tyr Gly His Ser Phe Gly Val Leu
325 330 335
Ser His Tyr Tyr Gly Arg Glu Ala Gly Leu Glu Leu Arg Glu Asp Ile
340 345 350
Asp Thr Ile Leu Glu Pro Gly Met Val Ile Ser Met Glu Pro Met Ile
355 360 365
Leu Ile Pro Glu Gly Gln Pro Gly Ala Gly Gly Tyr Arg Glu His Asp
370 375 380
Ile Leu Val Ile Gln Glu Asn Gly Val Val Glu Asp Ile Thr Gly Phe
385 390 395 400
Pro Phe Gly Pro Glu Tyr Asn Ile Ile Lys Lys
405 410

【0050】配列番号：2

配列の長さ：1236

配列の型：核酸

トポロジー：直鎖状

配列の種類：ゲノムDNA

＊ 起源

生物名：アースロバクター・エスピー(Arthrobacter s
p.)

株名：TE1826 (FERM P-10637)

＊

配列

ATG CAA AAA ATC ACT GAT CTT GAA AGA ACA AAA GTT CTG CAC AAT GCC 48
Met Gln Lys Ile Thr Asp Leu Glu Arg Thr Lys Val Leu His Asn Gly
1 5 10 15
GAA AAA TTT AAA GGT ACT TTC TCA AAA GCG GAA ATG GAC CGC AGG AAT 96
Glu Lys Phe Lys Gly Thr Phe Ser Lys Ala Glu Met Asp Arg Arg Asn
20 25 30
ACG AAC CTG CGT AAT TAC ATG GCT GAA AAA GAT ATT GAC GCT GTC CTA 144
Thr Asn Leu Arg Asn Tyr Met Ala Glu Lys Asp Ile Asp Ala Val Leu
35 40 45
TTT ACT TCT TAC CAC AAT ATT AAC TAT TAC AGC GAT TTC TTA TAT ACA 192
Phe Thr Ser Tyr His Asn Ile Asn Tyr Tyr Ser Asp Phe Leu Tyr Thr
50 55 60
TCT TTT AAC AGG AAT TAT CGA TTG GTT GTT ACC CAG AAC AAA CAC GTA 240
Ser Phe Asn Arg Asn Tyr Gly Leu Val Val Thr Gln Asn Lys His Val
65 70 75 80
ACA GTT AGT CCA AAC ATA GAT GCG GCG ATG CCT TCG AGA AGA AGC TAC 288
Thr Val Ser Ala Asn Ile Asp Gly Gly Met Pro Trp Arg Arg Ser Tyr
85 90 95
GAT GAA AAT ATT GTA TAC ACC GAC TGG AGA AGA GAC AAC TAT TTC TAT 336
Asp Glu Asn Ile Val Tyr Thr Asp Trp Arg Arg Asp Asn Tyr Phe Tyr
100 105 110
GCA ATT CAA AAA GTA CTA GAA GAA GCA GGA GTT AAG AAA GCC CGC TTA 384
Ala Ile Gln Lys Val Leu Glu Glu Ala Gly Val Lys Lys Ala Arg Leu
115 120 125
GGC ATT GAA GAG GAC CAT GTG TCC ATC GAT CTT CTG AGA AAA TTC TCA 432
Gly Ile Glu Glu Asp His Val Ser Ile Asp Leu Leu Arg Lys Phe Ser
130 135 140
GAC ACA TTT CCT AAC TTT GAA TTG GTT CAT GTT TCT CAA GAT GTT ATG 480
Asp Thr Phe Pro Asn Phe Glu Leu Val His Val Ser Gln Asp Val Met
145 150 155 160
AAA CAG CGG ATG ATC AAA TCT GCT GAG GAA ATT ACG CAT ATA AAA AAT 528
Lys Gln Arg Met Ile Lys Ser Ala Glu Glu Ile Arg His Ile Lys Asn

50 配列の種類：合成DNA

配列

AGGAA GGCAG GAGAT TAAGG ATGCA AAAAA TCACT GATC 49

【0052】配列番号：4

配列の長さ : 39

配列の型：核酸

* 鎖の数：一本鎖

トポロジー：直鎖状

* 配列の種類：合成DNA

配列

AATGG TGAAT TACTT TTTGA TAATA TTATA TTCAG GGCC 39

【図面の簡単な説明】

※【図2】 pCNA-1の制限酵素地図を示す図であ

【図1】本発明のクレアチンアミジノヒドロラーゼのア

る。

ミノ酸配列とシュードモナス・ブチダ (Pseudomonas pu 10) 【国】

【図3】 pCRHRA-1の制限酵素地図を示す図であ

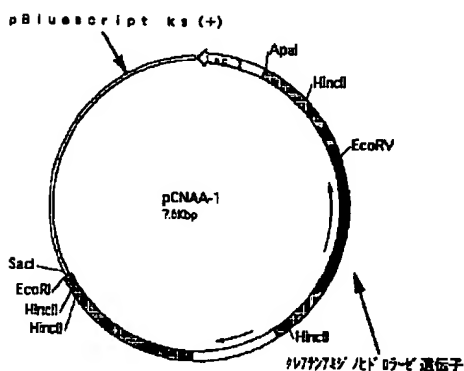
tida) が產生するクレアチンアミジノヒドロラーゼのア

る。

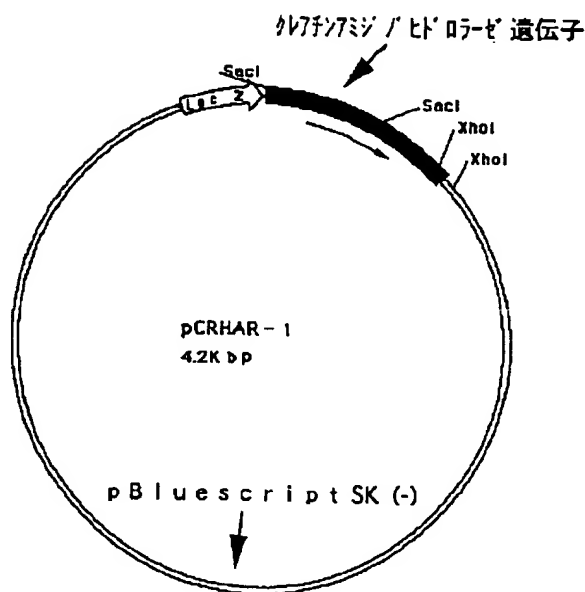
ミノ酸配列との比較を示す図である。 ※

✱

【圖2】



【圖3】



```

本特# 1' MOKITDLERTKVLHNGEKFKGTFKAEMDRRNTNLRNYMAEKDIDAVLFTSYHNINYYSDFLYTSFNRYGLVVQTQKHV
1" MQMEXTLIRNGDKVRSTFSAOEYANRQARLRAHLAAENIDAAIFTSYHNINYYSDFLYCSFGRPYALVVTTQDDVI

本特# 81' TVSANIDGGMPWRRSYD-ENIVYTDWRRDNFYAIQKVLEEAGVKKARLGIEDHVSIDLRLKFSDTFPNFELVHVSQDV
..***** ****. . ***** ***** ****. * ****. .... *... ..* .....
77" SISANIDGGQPWRRTVGTDNIVYTDWQRDNFYVAIQAL-----PKARRIGIEHDLNLQNRDKLAARYPDAELVDVAAAC

本特# 160' MKQRMKSAEIRHIIKNGARIADIGGYAVVEAIQGVPEYEVVALAGSKAMTREIAKLYPQSELRDTWVWFQAGINTDGAH
*.***** *..***** ***** ..***. ....*... ..*...*****.*****
153" MRMRKSAEBHVMIIRHGARIADIGGAHVVEALGDQVPEYEVVALHATQAMVRAIADTFEDVELMDTWTWFSQGINTDGAH

本特# 240' SWATSKKVQKGEILSLNTPFMIAGYYTALERTLFLSEVSDAHLKYWEINVEVHKRGLELIKPGAVCKDICAELENFRRH
..***. ....***** ***** ..***. *..***** ..***** *..*****. *
233" NPVTRKVNKGDILSLNCFPMIAGYYTALERTFLDHCSDHLRLWQNVVEVHEAGLKLKPGARCSDIARELNEIFLKH

本特# 320' DLVKNRTFGYGHSGFVLSHYGRAGLELRDIDITILEPGWVISMEPMILIPGQPGAGGYREHDILVIQENGVVEDITG
*... ***** ..***** ..***** ..***** ..***** ..***** ..*... ..*...
313" DVLQYRTFGYGHSGFGLSHYGRAGLELRDIDITVLEPGWVSMPEMIMLPEGLPGAGGYREHDILIVNENG-AENITK

本特# 400' FPFGEYNIIRK
**** *...
392" FPYGPEKNIIRK

```

フロントページの続き

(51)Int.Cl.⁶

識別記号

F I

C 1 2 R 1:06)

(C 1 2 N 1/21

C 1 2 R 1:19)

(C 1 2 N 9/80

C 1 2 R 1:06)

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(71)Applicant : TOYOBO CO LTD

(22)Date of filing : 08.04.1994

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KISHIMOTO TAKAHIDE
TEJIMA SHINICHI
KAWAMURA YOSHIHISA

(54) PRODUCTION OF CREATINE AMIDINOHYDROLASE

(57)Abstract:

PURPOSE: To provide a creatine amidinohydrolase useful as a clinical examination agent for the determination of creatinine and creatine by culturing a creatine amidinohydrolase-producing microorganism belonging to the genus *Paracoccus* or *Sphingobacterium*.

CONSTITUTION: This creatine amidinohydrolase is produced by culturing a creatine amidinohydrolase-producing microorganism belonging to the genus *Paracoccus* or *Sphingobacterium*, preferably *Paracoccus denitrificans* UNGS AND *var. amabushungs* *Paracoccus alkalophilus* JCM7364, *Sphingobacterium multivorum* TE3580, etc., in a nutrient medium and separating the produced enzyme from the cultured product.

LEGAL STATUS

[Date of request for examination] 30.03.2001

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partial translation (JP Publication No. 07-274961)

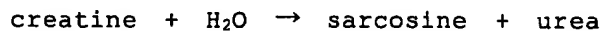
page 5, right column, lines 8-37

The obtained creatine amidinohydrolase showed the following properties.

1. Following reactions were catalyzed.

[0020]

[chemical 1]



[0021] 2. Km value: The Km value for creatine was about 22.8 mM.

3. Optimal pH: The enzymatic activity in 50 mM K-phosphate buffer (pH 6.0-8.0), 50 mM Tris-HCl buffer (pH 8.0-9.0), and glycine-NaOH buffer (pH 9.0-10.5) was measured. The results are as shown in Fig. 1. The optimal pH was about 8.0-9.5.

4. pH stability: The residual activity was measured after storage in glycine-HCl buffer (pH 2-3), acetate buffer (pH 3-6), K-phosphate buffer (pH 6-8), Tris-HCl buffer (pH 8-9), or glycine-NaOH buffer (pH 9-10) at 25°C for about 18 hr.

5. Optimal temperature: The enzymatic activity was measured at each temperature. The results are as shown in Fig. 3. The optimal temperature was about 35-40 °C.

6. Heat stability: The enzyme of the present invention was warmed in 50 mM K-phosphate buffer (pH 7.5) for 30 min and the residual enzymatic activity was measured. The results are as shown in Fig. 4. The enzyme was stable up to about 40°C.

7. Molecular weight: about 68,000 (gel filtration method)

about 50,000 (SDS-PAGE)

8. Isoelectric point: about 4.1 (isoelectric focusing)

(19)日本国特許庁 (J P)

(12) 公 開 特 許 公 報 (A)

(11)特許出願公開番号

特開平7-274961

(43)公開日 平成7年(1995)10月24日

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C 1 2 N 9/80		Z		
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C 1 2 R 1:01)				

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(22)出願日	平成6年(1994)4月8日	(72)発明者	服部 静夫 福井県敦賀市東洋町10番24号 東洋紡績株式会社敦賀バイオ研究所内
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最終頁に続く

(54)【発明の名称】 クレアチンアミジノヒドロラーゼの製造法

(57)【要約】

【目的】新規な菌株からクレアチンアミジノヒドロラーゼを製造する。

【構成】パラコッカス・デナイトリフィカンズ (Paracoccus denitrificans)、パラコッカス・アルカロフィラス (Paracoccus alkalophilus) またはスフィンゴバクテリウム・マルチボラム (Sphingobacterium multivorum) に属する菌株を培養して、クレアチンアミジノヒドロラーゼを採取する。

【特許請求の範囲】

【請求項1】 バラコッカス属またはスフィンゴバクテリウム属に属し、クレアチンアミジノハイドロラーゼを生産する能力を有する微生物を栄養培地に培養し、培養物中にクレアチンアミジノハイドロラーゼを生成蓄積せしめ、該培養物から該酵素を採取することを特徴とするクレアチンアミジノハイドロラーゼの製造法。

【請求項2】 バラコッカス属に属し、クレアチンアミジノハイドロラーゼを生産する能力を有する微生物が、
 バラコッカス・デナイトリフィカンス (Paracoccus denitrificans) またはバラコッカス・アルカロフィラス (Paracoccus alkalophilus) であることを特徴とする請求項1記載のクレアチンアミジノハイドロラーゼの製造法。

【請求項3】 バラコッカス属に属し、クレアチンアミジノハイドロラーゼを生産する能力を有する微生物が、
 バラコッカス・デナイトリフィカンス (Paracoccus denitrificans) IFO14907またはバラコッカス・アルカロフィラス (Paracoccus alkalophilus) JCM7364であることを特徴とする請求項1記載のクレアチンアミジノハイドロラーゼの製造法。

【請求項4】 スフィンゴバクテリウム属に属し、クレアチンアミジノハイドロラーゼを生産する能力を有する微生物が、スフィンゴバクテリウム・マルチボラム (Sphingobacterium multivorum) であることを特徴とする請求項1記載のクレアチンアミジノハイドロラーゼの製造法。

【請求項5】 スフィンゴバクテリウム属に属し、クレアチンアミジノハイドロラーゼを生産する能力を有する微生物が、スフィンゴバクテリウム・マルチボラム (Sphingobacterium multivorum) TE3580 (FERM P-14209) であることを特徴とする請求項1記載のクレアチンアミジノハイドロラーゼの製造法。

【発明の詳細な説明】

【0001】

【産業上の利用分野】 本発明はクレアチンアミジノハイドロラーゼの製造法に関する。

【0002】 クレアチニンおよびクレアチンは血液または尿中に見いだされ、その量を迅速かつ正確に検出測定することは疾病、例えば尿毒症、慢性腎炎、急性腎炎、巨人症、強直性筋異常栄養症等を診断するのに非常に重要である。このような診断を行うために、血液または尿中のクレアチニンおよびクレアチンを定量することが一般的に行われている。

【0003】

【従来の技術】 従来からクレアチンの定量法としては、試料中のクレアチンにクレアチンアミジノハイドロラーゼ、ザルコシンオキシダーゼを作用させ、生成する過酸化水素を過酸化水素検出手段により測定して、試料中のクレアチンを定量する方法があり、またクレアチニンの

定量法としては、試料中のクレアチニンにクレアチンアミドハイドロラーゼを作用させ、生成するクレアチンにクレアチンアミジノハイドロラーゼ、ザルコシンオキシダーゼを作用させて、試料中のクレアチニンを定量する方法がある。

【0004】 一方、これらの定量法に使用するクレアチンアミドハイドロラーゼ、クレアチンアミジノハイドロラーゼ、ザルコシンオキシダーゼは微生物界に広く見いだされており、特にクレアチンアミジノハイドロラーゼは、シュードモナス属、アースロバクター属、フラボバクテリウム属、マイクロコッカス属、アルカリゲネス属、ベニシリウム属、アシネトバクター属、コリネバクテリウム属、アクチノバチルス属、バチルス属細菌から得られることが報告されており、その中の幾つかは既に工業的に製造され、臨床検査薬として使用されている。

【0005】

【発明が解決しようとする課題】 本発明者らは上記細菌の他の菌株から、新しくクレアチンアミジノハイドロラーゼを製造することを試みた。

【0006】

【課題を解決するための手段】 本発明者らは種々検討した結果、新たにバラコッカス属またはスフィンゴバクテリウム属に属する菌株がクレアチンアミジノハイドロラーゼを生産することを見出し本発明に到達した。

【0007】 すなわち本発明はバラコッカス属またはスフィンゴバクテリウム属に属し、クレアチンアミジノハイドロラーゼを生産する能力を有する微生物を栄養培地に培養し、培養物中にクレアチンアミジノハイドロラーゼを生成蓄積せしめ、該培養物から該酵素を採取することを特徴とするクレアチンアミジノハイドロラーゼの製造法である。

【0008】 本発明に使用する微生物としては、バラコッカス属またはスフィンゴバクテリウム属に属し、クレアチンアミジノハイドロラーゼを生産する能力を有するものであれば、いずれの菌株を用いても良い。バラコッカス属に属する菌株としては、好ましくはバラコッカス・デナイトリフィカンス (Paracoccus denitrificans)、バラコッカス・アルカロフィラス (Paracoccus alkalophilus) などが挙げられる。スフィンゴバクテリウム属に属する菌株としては、スフィンゴバクテリウム・マルチボラム (Sphingobacterium multivorum) などが挙げられる。

【0009】 更により好ましくはバラコッカス・デナイトリフィカンス (Paracoccus denitrificans) IFO14907、バラコッカス・アルカロフィラス (Paracoccus alkalophilus) JCM7364、スフィンゴバクテリウム・マルチボラム (Sphingobacterium multivorum) TE3580などが挙げられる。なお、スフィンゴバクテリウム・マルチボラム (Sphingobacterium multivorum) TE3580は、京都市左京区深泥池の土壌から分

離した菌株であり、その菌学的性質は以下の通りである。

【0010】(a) 形態

- (1) 菌形：かん菌
- (2) 細胞の大きさ：0.5×1.5~2.5μm
- (3) 細胞の多形性：無し
- (4) 運動性：無し
- (5) 胞子の有無：無し

(b) 各培地における生育状態

- (1) 肉汁寒天平板培地：30℃、48時間培養でロウ 10
色のコロニーを形成する。コロニーの周縁は全縁(Entire)であり、クッション状(Pulvinate)である。表面は円滑(smooth)で光沢を有し、不透明である。
- (2) 肉汁液体培養：生育は普通で一様に混濁する。
- (3) 肉汁ゼラチン穿刺培養：生育は普通で上部のみ糸状(Filiform)に生育する。ゼラチンは液化しない。
- (4) リトマスミルク：色に変化はない。ミルクは固化しない。
- (5) マッコンキー寒天培地：生育しない。
- (6) フェニルエチルアルコール寒天培地：生育しない。 20

【0011】(C) 生理学的性質

- (1) グラム染色性： - (陰性)
- (2) 色素の生成：黄土色の色素を生成する。
- (3) 硝酸塩の還元： -
- (4) 脱窒反応： -
- (5) MRテスト： -
- (6) VPテスト： -
- (7) インドールの生成： -
- (8) 硫化水素の生成： - 30
- (9) デンプンの加水分解： -

(10) Tween 80の分解： -

(11) クエン酸の利用：Koserの培地 -、Christensenの培地 -

(12) 無機窒素源の利用：

硝酸ナトリウム +
硫酸アンモニウム +
グルタミン酸ナトリウム +

(13) ウレアーゼ： +

(14) オキシダーゼ： +

(15)カタラーゼ： +

(16)β-ガラクトシダーゼ： +

(17) アルギニンジヒドラーゼ： -

(18) リジンカルボキシラーゼ： -

(19) オルニチンカルボキシラーゼ： -

(20) トリプトファンデアミナーゼ： +

(21)β-グルコシダーゼ： +

(22) プロテアーゼ： -

(23) DNase： +

(24) 生育の範囲：

生育温度	20℃	+
	30℃	+
	37℃	-
	40℃	-
	50℃	-
生育pH	pH4	-
	pH7	+
	pH9	+

(25) 酸素に対する態度：好気性

(26) O-Fテスト(Hugh Leifson法)：O(酸化)

(27) 糖から酸およびガスの生成

	酸	ガス
L-アラビノース	+	-
D-キシロース	+	-
D-グルコース	+	-
D-マンノース	+	-
D-フラクトース	+	-
D-ガラクトース	+	-
マルトース	+	-
シュークロース	-	-
ラクトース	+	-
トレハロース	+	-
D-ソルビトール	-	-
D-マンニトール	-	-
イノシトール	-	-
グリセリン	-	-
デンプン	-	-
ラムノース	-	-
D-メリビオース	-	-
D-アミダクリン	-	-

(28) 有機化合物の利用

D-グルコース	+
L-アラビノース	+
D-マンノース	+
D-マンニトール	+
N-アセチル-D-グルコサミン	+
マルトース	+
グルコン酸カリウム	-
n-カプリン酸塩	-
アジピン酸	-
d1-リンゴ酸	-
クエン酸ナトリウム	-
酢酸フェニル	-

考えられ、スフィンゴバクテリウム・マルチボラム (*Sphingobacterium multivorum*) TE 3580 と命名した。本菌は受託番号、FERM P-14209 として寄託されている。

【0013】本発明の酵素を製造するにあたっては、上記クレアチンアミジノヒドロラーゼ生産菌を栄養培地に培養し、該培養物からクレアチンアミジノヒドロラーゼを採取することにより製造できる。クレアチンアミジノヒドロラーゼ生産菌の培養にあたって使用する培地としては、使用菌株が資化しうる炭素源、窒素源、無機物、その他必要な栄養素を適量含有するものであれば、合成培地、天然培地いずれも使用できる。炭素源としては、例えばグルコース、グリセロール酸等が使用される。窒素源としては、例えばペプトン類、肉エキス、酵母エキス等の窒素含有天然物や、塩化アンモニウム、クエン酸アンモニウム等の無機窒素含有化合物が使用される。無機物としては、リン酸カリウム、リン酸ナトリウム、硫酸マグネシウム等が使用される。またクレアチンアミジノヒドロラーゼの生産誘導物質として、クレアチンを培地に添加しておくことが望ましい。

【0014】本発明では通常、振盪培養あるいは通気攪拌培養を行う。培養温度は約20~45℃、好ましくは約25~37℃、培養pHは約5~9の範囲で、好ましくは約6~8に制御するのが良い。これら以外の条件下でも使用する菌株が生育すれば実施できる。培養期間は通常、約1~7日で生育し、菌体内にクレアチンアミジノヒドロラーゼが生産蓄積される。

【0015】本発明の酵素の精製法は一般に使用される精製法を用いれば良い。例えば抽出法としては超音波破碎、ガラスビーズを用いる機械的な破碎、フレンチブレ

【0012】上記菌学的性質同定のための実験法は、主として長谷川武治編著、改訂版「微生物の分類と同定」学会出版センター(1985年)によって行った。また分類同定の基準として、「バージェーズ・マニュアル・オブ・システムチック・バクテリオロジー」(1984)およびインターナショナル・ジャーナル・オブ・システムチック・バクテリオロジー Vol. 33, 580 (1983)を参考にした。以上の文献および菌学的性質を参考にするとグルコース非発酵性グラム陰性かん菌で黄土色の色素を生成すること、オキシダーゼ、カタラーゼ、β-グルコシダーゼ、β-ガラクトシダーゼ、ウレアーゼが陽性、各種糖類より酸を生成することより、本菌はスフィンゴバクテリウム (*Sphingobacterium*) 属に属するとみなされる。またスフィンゴバクテリウム中ではデンプンを分解しないこと、マンニトールより酸を生成しないことを考えるとスフィンゴバクテリウム・マルチボラム (*Sphingobacterium multivorum*) に属すると

ス、界面活性剤などいずれを用いても良い。さらに抽出液については、硫酸やばう硝などの塩析法、塩化マグネシウムや塩化カルシウムなどの金属凝集法、プロタミンやポリエチレンイミンなどの凝集法、さらにはDEAE（ジエチルアミノエチル）セファロース、CM（カルボキシメチル）セファロースなどのイオン交換クロマト法などにより精製することができる。またこれらの方法で得られた粗酵素液や精製酵素液は、例えばスプレードライや凍結乾燥により粉末化できる。さらには適当な担体に固定化して固定化酵素として使用できる。

【0016】本発明の製造法により得られたクレアチンアミジノヒドロラーゼは、クレアチンに作用して、ザルコシンおよび尿素を生産する。このクレアチンアミジノヒドロラーゼは、ザルコシンオキシダーゼおよび過酸化水素検出組成物と組み合わせてクレアチンの測定に使用することができる。またクレアチンアミドヒドロラーゼを共存させて、クレアチンを定量することができる。

【0017】次に本発明のクレアチンアミジノヒドロラーゼの活性測定法を示す。まず試験管に基質溶液（クレアチンを0.1Mとなるように50mMリン酸緩衝液、pH7.5に溶解したもの）0.9mlをとり、37℃で約5分予備加温する。次に酵素溶液0.1mlを加え、反応を開始し、37℃で正確に10分間反応させた後、DAB溶液（2.0gのジメチルアミノベンズアルデヒドを100mlのジメチルスルホキシドに溶解させた後、濃塩酸15mlを加える。）2.0mlを加えて反応を停止させる。25℃で20分間放置後、生成した尿素がジメチルアミノベンズアルデヒドと縮合して生成した黄色色素（Ehrich反応生成物）を425nmにおける吸光度で測定する。盲検は基質溶液0.9mlを37℃で10分間放置後、DAB溶液2.0mlを加えて混和し、ついで酵素溶液0.1mlを加えて調製し、以下同様に25℃で20分間放置後、吸光度を測定する。上記条件下で1分間に1マイクロモルの黄色色素を生成する酵素量を1単位（U）とする。

【0018】

【実施例】以下、実施例を挙げて本発明を具体的に示す。

実施例1

クレアチン1%、ポリペプトン1%、酵母エキス0.5%、NaCl0.5%を含む培地（pH7.0）100mlを500ml容坂口フラスコに移し、121℃、15分間オートクレーブを行った。種菌として、パラコッカス・デナイトリフィカンス（*Paracoccus denitrificans*）IFO14907を一白金耳植菌し、30℃で時間培養し、種培養液とした。次に同培地6リットルを10リットル容ジャーフェーマンターに移し、121℃で15分間オートクレーブを行い、放冷後、種培養液100mlを移し、300rpm、通気量2l/分、30℃で

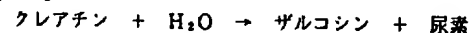
時間培養した。培養液を遠心分離にて集菌し、50mMリン酸緩衝液（pH7.0）に懸濁した。

【0019】上記菌体懸濁液をフレンチプレスで処理し、遠心分離を行い、上清液を得た。得られた粗酵素液を硫酸分画、DEAEセファロースクロマトグラフィー、フェニルセファロースクロマトグラフィー、セファデックスG-200によるゲルろ過により比活性、約18U/mgにまで精製した。得られたクレアチンアミジノヒドロラーゼは下記特性を有していた。

1. 下記の反応を触媒した。

【0020】

【化1】



【0021】2. Km値：クレアチンに対するKm値は約22.8mMであった。

3. 至適pH：50mM K-リン酸緩衝液（pH6.0～8.0）、50mMトリス塩酸緩衝液（pH8.0～9.0）、グリシンNaOH緩衝液（pH9.0～10.5）中での酵素活性を測定した。その結果は図1に示す通りであって、至適pHは約8.0～9.5であった。

4. 安定pH：グリシン塩酸緩衝液（pH2～3）、酢酸緩衝液（pH3～6）、K-リン酸緩衝液（pH6～8）、トリス塩酸緩衝液（pH8～9）、グリシンNaOH緩衝液（pH9～10）で25℃、約18時間保存してその残存活性を測定した。その結果は図2に示す通りであって、安定pHはpH約4～10であった。

5. 至適温度：各温度における酵素活性を測定した。その結果は図3に示す通りであって、至適温度は約35～40℃であった。

6. 熱安定性：本発明の酵素を50mM K-リン酸緩衝液（pH7.5）中で30分間保温した後、残存する酵素活性を測定した。その結果は図4に示す通りであって、約40℃まで安定であった。

7. 分子量：約68,000（ゲル濾過法）

約50,000（SDS-PAGE）

8. 等電点：約4.1（等電点電気泳動）

【0022】実施例2

クレアチン1%、ポリペプトン1%、酵母エキス0.5%、NaCl0.5%を含む培地（pH7.0）100mlを500ml容坂口フラスコに移し、121℃、15分間オートクレーブを行った。種菌として、パラコッカス・アルカロフィラス（*Paracoccus alkalophilus*）JCM7364を一白金耳植菌し、30℃で時間培養し、種培養液とした。次に同培地6リットルを10リットル容ジャーフェーマンターに移し、121℃で15分間オートクレーブを行い、放冷後、種培養液100mlを移し、300rpm、通気量2l/分、30℃で時間培養した。培養液を遠心分離にて集菌し、50mMリン酸緩衝液（pH7.0）に懸濁し、フレンチプレスで処理

し、遠心分離を行い、上清液を得た。得られた粗酵素液は約0.3 U/mlのクレアチンアミジノヒドロラーゼを含有していた。

【0023】実施例3

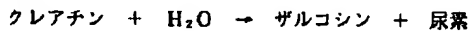
クレアチン1%、ポリペプトン1%、酵母エキス0.5%、NaCl0.5%を含む培地(pH7.0)100 mlを500 ml容坂口フラスコに移し、121℃、15分間オートクレーブを行った。種菌として、スフィンゴモナス・マルチボラム(*Sphingobacterium multivoru* m)TE3580(FERM P-14209)を一白金耳植菌し、30℃で時間培養し、種培養液とした。次に同培地6リットルを10リットル容ジャーファーマンターに移し、121℃で15分間オートクレーブを行い、放冷後、種培養液100 mlを移し、300 rpm、通気量2 l/分、30℃で時間培養した。培養液を遠心分離にて集菌し、50 mMリン酸緩衝液(pH7.0)に懸濁した。

【0024】上記菌体懸濁液をフレンチプレスで処理し、遠心分離を行い、上清液を得た。得られた粗酵素液を硫酸分画、DEAE-セファロースクロマトグラフィー、フェニルセファロースクロマトグラフィー、セファデックスG-200によるゲルろ過により比活性約21 U/mgにまで精製した。得られたクレアチンアミジノヒドロラーゼは下記特性を有していた。

1. 下記の反応を触媒した。

【0025】

【化2】



2. Km値：クレアチンに対するKm値は約18.5 mMであった。

3. 至適pH：50 mM K-リン酸緩衝液(pH5.5~8.0)、50 mMトリス塩酸緩衝液(pH7.5~8.5)、グリシンNaOH緩衝液(pH9.0~10.5)中での酵素活性を測定した。その結果は第5図に示す通りであって、至適pHは約7.5~9.0であった。

4. 安定pH：グリシン塩酸緩衝液(pH2~3)、酢

酸緩衝液(pH3~6)、K-リン酸緩衝液(pH6~8)、トリス塩酸緩衝液(pH8~9)、グリシンNaOH緩衝液(pH9~10)で、25℃、18時間保存してその残存活性を測定した。その結果、安定pHは約4~10であった(図6)。

5. 至適温度：各温度における酵素活性を測定した。その結果は図7に示す通りであって、至適温度は約40℃であった。

6. 熱安定性：本発明の酵素を50 mM K-リン酸緩衝液(pH7.5)中で30分間保温した後、残存する酵素活性を測定した。その結果は図8に示す通りであって、45℃まで安定であった。

7. 分子量：約73,000(ゲル濾過法)

約44,000(SDS-PAGE)

【0026】

【発明の効果】本発明の製造法により、今まで未報告の菌株であるバラコッカス属またはスフィンゴバクテリウム属に属する菌株からクレアチンアミジノヒドロラーゼを得ることができる。

【図面の簡単な説明】

【図1】バラコッカス属の酵素の反応pHと相対活性との関係を示すグラフである。

【図2】バラコッカス属の酵素のpH安定性を示すグラフである。

【図3】バラコッカス属の酵素の反応温度と相対活性との関係を示すグラフである。

【図4】バラコッカス属の酵素の熱安定性を示すグラフである。

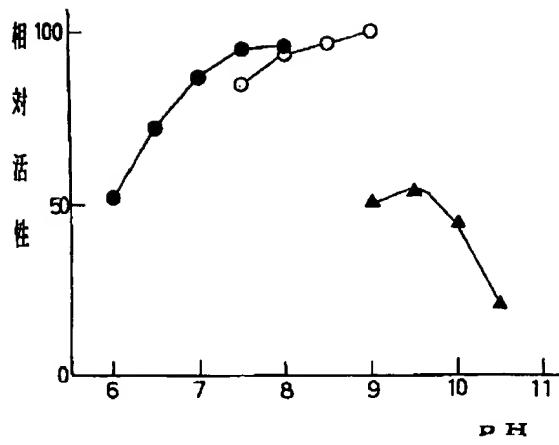
【図5】スフィンゴバクテリウム属の酵素の反応pHと相対活性との関係を示すグラフである。

【図6】スフィンゴバクテリウム属の酵素のpH安定性を示すグラフである。

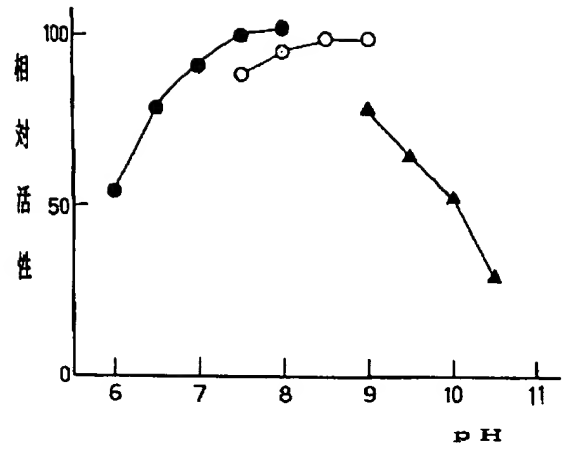
【図7】スフィンゴバクテリウム属の酵素の反応温度と相対活性との関係を示すグラフである。

【図8】スフィンゴバクテリウム属の酵素の熱安定性を示すグラフである。

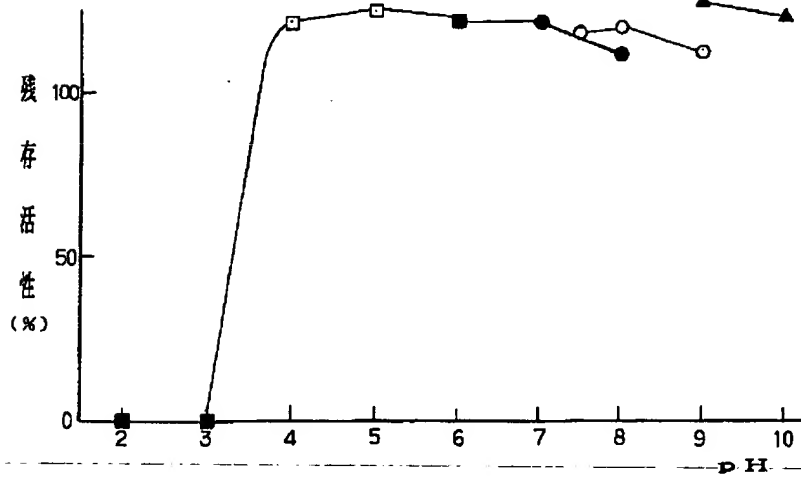
【図1】



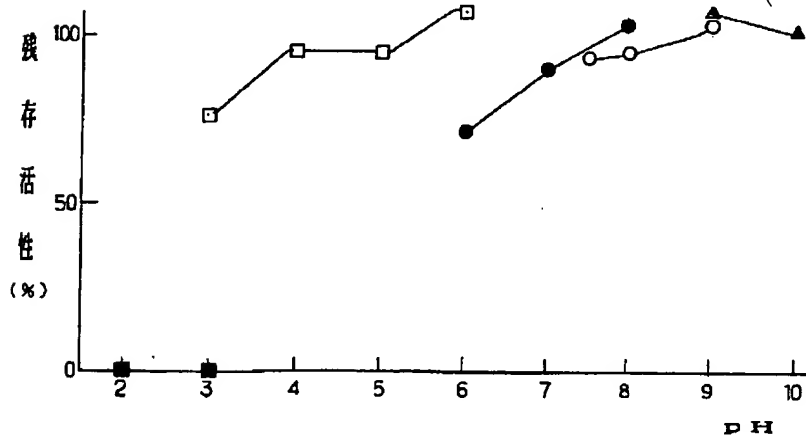
【図5】



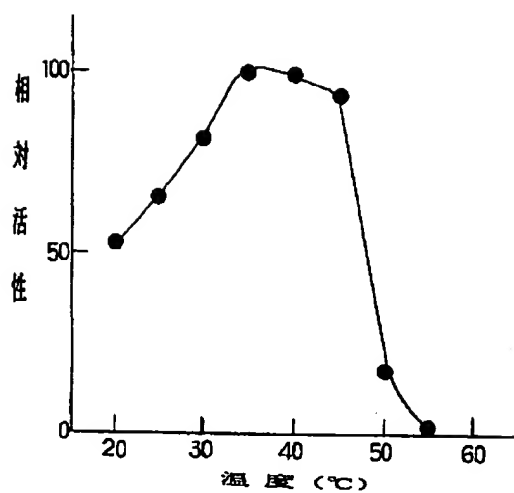
【図2】



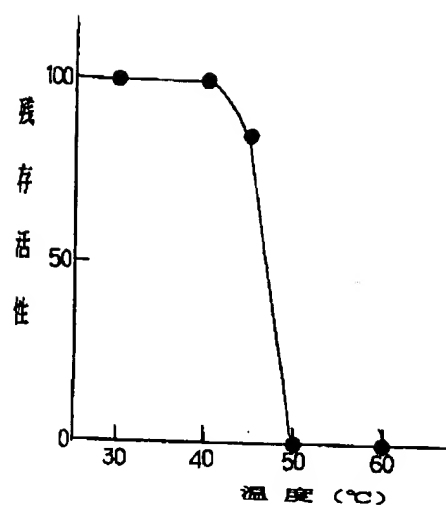
【図6】



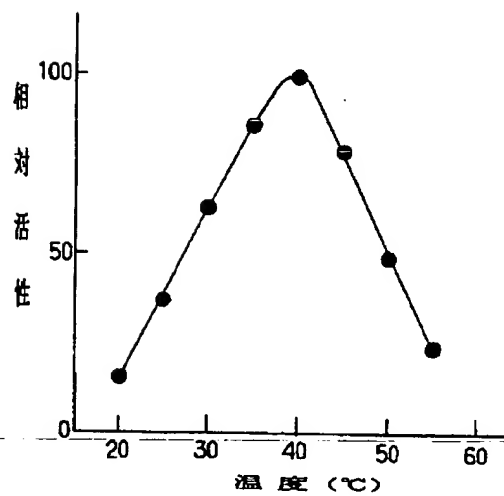
【図3】



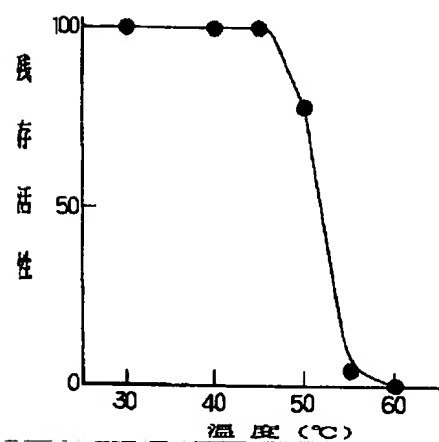
【図4】



【図7】



【図8】



フロントページの続き

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United States Patent [19]

Ikuta et al.

[11] **4,420,562**[45] **Dec. 13, 1983**[54] **METHOD FOR PRODUCING CREATINASE**[75] **Inventors:** Shigeru Ikuta; Kazuo Matsuura;
Hideo Misaki, all of Shizuoka, Japan[73] **Assignee:** Toyo Jozo Company, Ltd., Tokyo,
Japan[21] **Appl. No.:** 371,458[22] **Filed:** Apr. 23, 1982**Related U.S. Application Data**[63] Continuation-in-part of Ser. No. 338,607, Jan. 11, 1982,
abandoned, which is a continuation-in-part of Ser. No.
158,800, Jun. 12, 1980, abandoned.[30] **Foreign Application Priority Data**

Jul. 4, 1979 [JP] Japan 54-85260

[51] **Int. Cl.³** C12N 9/78; C12R 1/07[52] **U.S. Cl.** 435/227; 435/815;
435/832[58] **Field of Search** 435/227, 221

[56]

References Cited**U.S. PATENT DOCUMENTS**

3,907,644	9/1975	Mollering et al.	435/227 X
4,039,384	8/1977	Suzuki et al.	435/227
4,216,292	8/1980	Ikuta et al.	435/191

Primary Examiner—Lionel M. Shapiro*Attorney, Agent, or Firm*—Wyatt, Gerber, Shoup,
Scobey & Badie

[57]

ABSTRACT

Isolation method of creatinase is disclosed. The method comprises culturing a microorganism belonging to *Bacillus*, for example, B-0618 strain (deposition No. FERM-P 4049 at Fermentation Research Institute, Agency of Industrial Science and Technology, Japan) to obtain cells from the cultured product, obtaining sarcosine oxidase and creatinase-containing solution, fractionally eluting sarcosine oxidase and creatinase by anion exchange chromatography to obtain a creatinase fraction and then collecting creatinase.

3 Claims, 4 Drawing Figures

FIG. 2

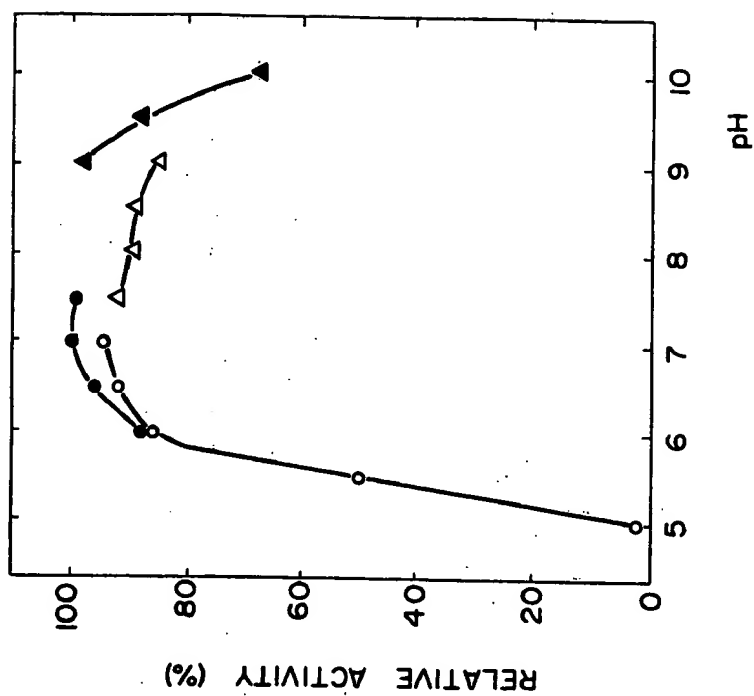


FIG. 1

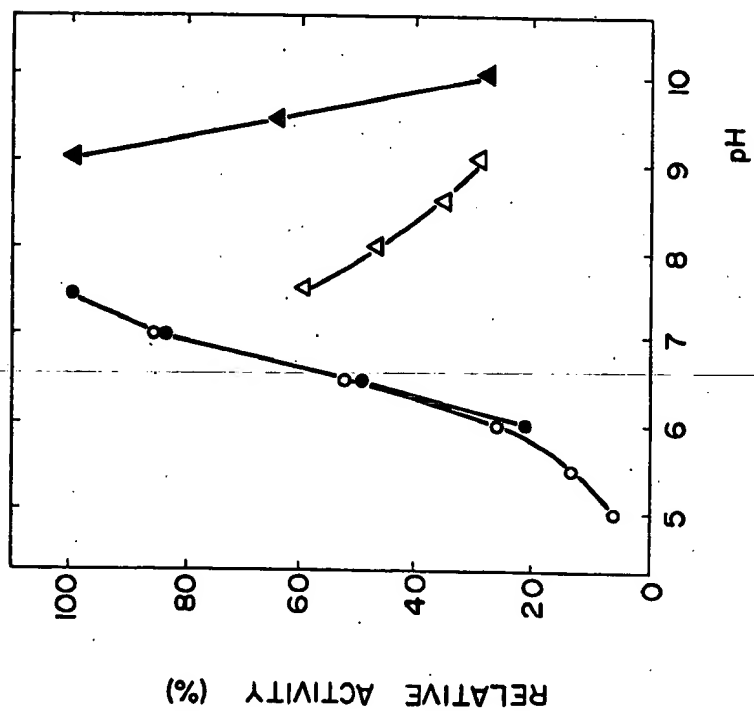


FIG. 4

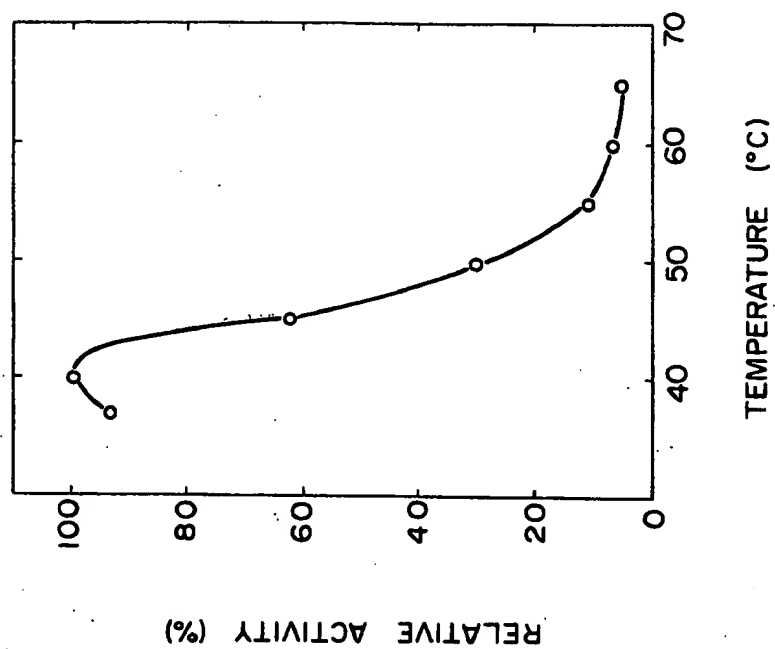
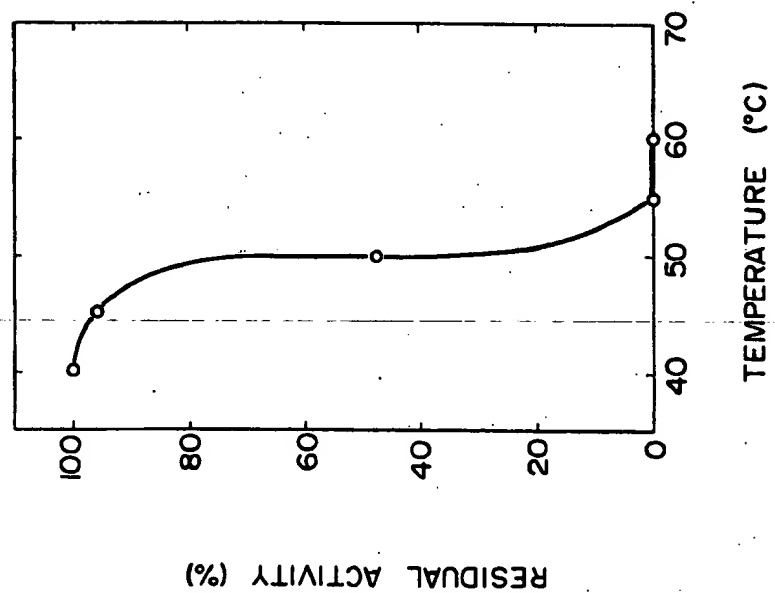


FIG. 3

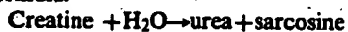


METHOD FOR PRODUCING CREATINASE

This application is a continuation-in-part application of application Ser. No. 338,607 filed on Jan. 11, 1982, abandoned which is a continuation-in-part application of Ser. No. 158,800, filed June 12, 1980, now abandoned.

The present invention pertains to the method for producing and isolating creatinase.

Creatinase is an enzyme classified as creatine amidinohydrolase with the enzyme number of 3.5.3.3., catalyzing the reaction represented by the following formula.



The production of the enzyme has so far been confined to microorganisms belonging to the genera *Pseudomonas*, *Flavobacterium*, etc.

It has now been found that a microorganism, B-0618 strain, belonging to the genus *Bacillus*, which was isolated from a soil sample taken from an egg-plant farm in Shimosasaki, Fukuchiyama City, Kyoto Prefecture, Japan, is capable of producing cellular creatinase from which it can be isolated successfully, when the strain is cultured in a medium containing a large amount of creatine.

The characteristics of the culture of the B-0618 strain on various culture media, according to the observations with naked eye and microscope, are described in detail in U.S. Pat. No. 4,216,292. The sarcosine oxidase and creatinase-producing B-0618 strain was named *Bacillus*, sp. B-0618, deposited with the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, and assigned the number, FERM-P No. 4049.

The present invention relates to a method for producing and isolating creatinase which comprises culturing *Bacillus* sp. B-0618, FERM-P No. 4049 which is a sarcosine oxidase and creatinase-producing strain of the genus *Bacillus* in a culture medium, obtaining cells from the cultured product, disrupting the sarcosine oxidase and creatinase-containing solution, and subjecting the solution to fractional elution by anion exchange chromatography to obtain a creatinase fraction and collecting creatinase.

More particularly, the present invention relates producing and isolating creatinase which comprises subjecting the sarcosine oxidase and creatinase-containing solution obtained above to elution by concentration gradient of KCl 0.1 M to 0.5 M solutions by anion exchange chromatography, e.g., diethylaminoethyl cellulose to recover the fraction eluted at KCl 0.3 M concentration and collecting creatinase.

The microorganism employed in the present invention, mention may be made of *Bacillus* sp. B-0618 strain as described above. Variants may be produced naturally or artificially. Any of such variants may be employed in the present invention so long as they retain the capability of producing creatinase.

In practicing the present invention, the *Bacillus* sp. B-0618 is cultivated according to the conventional procedure for producing antibiotics, enzymes, etc. The type of the cultivation may be either liquid or of solid. From the industrial point view, however, it is advantageous that cells of the present strain is inoculated to a industrial scale culture medium, to effect submerged cultivation under aeration-agitation.

Conventional nutrient sources may be utilized. Typical nitrogen compounds which may be used as the nitrogen source include corn steep liquor, soybean powder, peptone, various meat extracts, yeast extract, ammonium sulfate, ammonium chloride, and the like. Useful compounds which may be used as the carbon source include glucose, molasses, glycerol, starch hydrolyzate, and the like. If necessary, salts, such as sodium chloride, potassium chloride, magnesium sulfate, potassium dihydrogen phosphate, potassium monohydrogen phosphate, and the like, may also be added. In order to produce creatinase, it is necessary that creatine be present in the culture medium. When the culture medium contains a small amount of creatine, sarcosine oxidase is predominantly produced, but when the culture medium contains a large amount of creatine, creatinase is predominantly produced. It is, therefore, preferable that creatine be added to the culture medium in order to increase the creatinase production during the cultivation. The preferred amount of creatine to be added ranges approximately from 1.5 to 2%, thereby a remarkably high yield of creatinase can be obtained.

The cultivation temperature may be suitably selected within the temperature range where creatinase is produced, but a temperature of from 26° to 33° C. is particularly advantageous. The period of the cultivation varies depending upon the various conditions, but 15 to 25 hours cultivation is ordinarily sufficient. The cultivation may be terminated when the potency of creatinase activity reaches the maximum. Creatinase and sarcosine oxidase are contained or accumulated in the cells in the cultivated product thus obtained.

The method of obtaining crude sarcosine oxidase and creatinase solution through extraction or elution of sarcosine oxidase and creatinase from the cultivated product, is exemplified hereunder. The cultivated product is subjected to solid-liquid separation, and the resulting wet cells are suspended in a phosphate buffer or Tris-HCl buffer, according to choice. Then, sarcosine oxidase and creatinase are extracted from the cells according to suitably selected or combined processes for enzyme extraction from cells, such as lysozyme processing, supersonic processing, French press processing, etc., to obtain a crude sarcosine oxidase and creatinase containing solution.

Purified creatinase may be obtained from the crude sarcosine oxidase and creatinase solution by conventional purification processes utilized for proteins and enzymes. For instance, the crude sarcosine oxidase and creatinase solution may be mixed with an aqueous protamine sulfate solution to remove nucleic acids, and then mixed with an organic solvent, such as acetone, methanol, ethanol, isopropanol, etc., for fractional precipitation, or with a similar salt such as ammonium sulfate, for salting-out of the enzyme, thereby to obtain the desired precipitates. The recovered precipitates may be purified, for instance, until they show a homogeneous protein band in electrophoresis technique. The methods of purification is advantageously one which utilizes the characteristics of creatinase. For instance, the precipitates may be dissolved in a medium such as Tris-HCl buffer, and subjected to a chromatography by use of an anion-exchanging substance, such as diethylaminoethyl-cellulose, crosslinked diethylaminoethyl dextran gel, crosslinked diethylaminoethyl agarose gel, etc. or a gel-filtration agent, such as dextran gel and polyacrylamide gel. For example, a chromatography method with an anion exchanger of diethylaminoethyl cellulose may be

used to obtain a fraction eluted with 0.3 M KCl solution in a concentration gradient of 0.1 to 0.5 M. According to the chromatography method by use of diethylaminoethyl cellulose, creatinase is eluted with 0.3 M KCl solution, while sarcosine oxidase is eluted with 0.36 M KCl solution. KCl concentration suitable for elution varies depending on the anion exchanging substance for chromatography employed. No doubt, the purification may be performed by a suitable combination of these procedures. Finally, purified powder containing only creatinase can be obtained by drying the product, for example, by lyophilization freeze drying process.

In the accompanying drawings,

FIG. 1 represents the optimum pH dependence of creatinase;

FIG. 2, pH stability of creatinase;

FIG. 3, thermal stability of creatinase; and

FIG. 4, the optimum temperature of creatinase activity.

Creatinase as obtained according to the present invention was assayed by the method of enzyme activity determination, and proved to have the physico-chemical properties, described below.

(1) Assay method of enzyme activity

Into 0.5 ml of a reaction mixture, consisting of 0.05 ml of 0.2 M phosphate buffer (pH 7.5) and 0.45 ml of 50 mM creatinase solution, is added 10 μ l of enzyme solution, and the mixture is incubated at 37° C. for 5 minutes. Thereafter, the reaction is stopped by adding 0.5 ml of a 0.5 mM PCMB solution, and sarcosine formed by the reaction is determined by using sarcosine oxidase (E.C.1.5.3.1). Thus, 0.5 ml of a reagent solution, consisting of 0.1 ml of 0.2 M Tris-HCl buffer (pH 8.0), 0.05 ml of 0.3% 4-aminoantipyrine solution, 0.05 ml of 0.2% phenol solution, 0.05 ml of 0.05% (W/V) peroxidase solution, 0.1 ml of sarcosine oxidase solution (30 U/ml), and 0.15 ml of distilled water, is added to the reaction mixture as mentioned above, and the mixture is incubated at 37° C. for 20 minutes. After addition of 1.5 ml of distilled water, the reaction mixture is determined colorimetrically at 500 nm, to estimate the amount of the generated hydrogen peroxide, from which the amount of sarcosine formed by the action of creatinase is calculated.

One unit (1 U.) of the enzyme activity is defined as the amount of enzyme which forms 1 μ mole of sarcosine from creatine at 37° C. per 1 minute.

(2) Reaction

The enzyme catalyzes the reaction which forms 1 mole of urea and sarcosine, from 1 mole of creatine with consumption of 1 mole of water.

(3) Optimum pH

The optimum pH of creatinase was found to be approximately 7.5-9.0. (Cf. FIG. 1)

The buffer solutions employed were dimethylglutarate-NaOH buffer (pH 5.0-7.0), phosphate buffer (pH 6.0-7.5), Tris-HCl buffer (pH 7.5-9.0), and glycine-NaOH buffer (pH 9.0-10.0), as shown in FIG. 1.

(4) pH stability

The enzyme was mixing with each of several buffers having different pH, incubated at 37° C. for 60 minutes, and thereafter assayed for residual activity. The buffers used were the same as described above. Creatinase is stable at about 6.0-9.0, as shown in FIG. 2.

(5) Thermal stability

Separate 1 ml fractions of creatinase solution in 10 mM phosphate buffer (pH 7.5) were subjected to various temperatures for 10 minutes, and assayed for the

residual activity according to the assay method based on enzyme activity as described above. Creatinase proved to be stable at temperatures below approximately 40° C., as shown in FIG. 3.

(6) Optimum reaction temperature

The optimum reaction temperature of creatinase was found to be approximately 40° C., as shown in FIG. 4.

(7) Molecular weight

About 74,000 (determined by gel filtration method).

(8) Isoelectric point

Around pH 4.9 (determined by isoelectric focusing electrophoresis, using a carrier ampholyte).

As mentioned heretofore, the enzyme, creatinase, of the present invention is recognized as an enzyme classified as creatine amidinohydrolase with the enzyme number of 3.5.3.3., from the fact that it reacts with creatine to form urea and sarcosine with consumption of water.

Creatinase of the invention can be utilized in various ways, including use as an enzymatic reagent for clinical diagnostics. For instance, it may be used for the determination of creatinine in serum or urine, in combination with creatinase and sarcosine oxidase, or it may be used for the assay of creatinase.

The following non-limiting examples are given by way of illustration only.

EXAMPLE 1

One hundred milliliters of a culture medium (pH 7.0), consisting of 0.5% of creatine, 0.5% of fish solubles, 0.2% of yeast extract, 0.3% of KCl, 0.1% of K_2HPO_4 , and 0.05% of $MgSO_4 \cdot 7H_2O$, were placed in a 500 ml volume Erlenmeyer flask, and sterilized at 120° C. for 20 minutes. The microorganism comprising the strain of *Bacillus* sp. B-0618, FERM-P No. 4049 was inoculated to the culture medium and cultivated at 30° C. for 1 day, thus obtaining a seed culture. Twenty liters of the culture medium having the same composition as described above was placed in a 30 l volume jar fermenter, and sterilized. After transplantation of the seed culture, the medium was cultivated under aeration-agitation at a temperature of 30° C. for 20 hours, with 200 r.p.m. stirring and 20 l/min. sterilized air rate. The resulting broth or cultivated product, was centrifuged to collect 12 g of wet cells, which were rinsed with a 10 mM phosphate buffer (pH 7.0), suspended in the same buffer, put with lysozyme (final concentration=0.2 mg/ml), incubated at 37° C. for 30 minutes, and centrifuged at 5,000 r.p.m. for 15 minutes, to obtain the supernatant (the enzyme activity as sarcosine oxidase, 1400 U and the enzyme activity as creatinase, 800 U). The resulting supernatant was mixed with 2.5 ml of an aqueous 2% protamine sulfate solution and centrifuged to remove nucleic acids. Then, the solution was treated with a saturated ammonium sulfate solution, and the precipitates settling at the fractions of from 50% to 70% concentration of ammonium sulfate, were separated and dissolved into 20 ml of 10 mM Tris-HCl buffer (pH 8.0). The fractionation with ammonium sulfate solution was repeated twice, and the precipitates were dissolved into 10 mM Tris-HCl buffer (pH 8.0). The resulting solution was desalted by passing through a column (3.5 cm diameter \times 30 cm) filled with dextran gel ("Cephadex G-25," made by Pharmacia), and charged into diethylaminoethyl ("DEAE-cellulose" made by Brown) column (2.0 cm diameter \times 18 cm), which had been equilibrated with 10 mM Tris-HCl buffer (pH 8.0). After washing with the same buffer containing 0.1 M KCl, the

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enzyme was eluted with aqueous KCl solutions in concentration gradient from 0.1 M to 0.5 M. The active fraction eluted with 0.3 M KCl solution and the active fraction eluted with 0.36 M KCl solution were separately recovered and the active fraction eluted with 0.3 M KCl solution was concentrated by a ultrafilter (made by Amicon), dialyzed for 10 hours against a 10 mM phosphate buffer (pH 7.5), and then lyophilized, thus obtaining creatinase powder (220 U of total activity, 38 mg of protein, 5.8 U/mg of specific activity, and 27.5% of recovery; sarcosine oxidase activity was not detected).

The active fraction eluted with 0.36 M KCl solution was dialyzed for 10 hours against a 10 mM Tris-HCl buffer (pH 8.0) by a dialysis membrane (Visking Co.) and then was freeze-dried to obtain sarcosine oxidase powder (540 U of total activity, 43 mg of protein, 12.7 U/mg of specific activity and 38.6% of recovery; creatinase activity was not detected.)

EXAMPLE 2

One hundred milliliters of a culture medium (pH 7.2) consisting of 1.5% creatine, 0.5% of peptone, 0.2% of yeast extract, 0.5% of corn steep liquor, 0.1% of KCl, 0.1% of K_2HPO_4 and 0.05% of $MgSO_4 \cdot 7H_2O$ were placed in a 500 ml volume Erlenmeyer flask, and sterilized at 120° C. for 20 minutes. Then, *Bacillus* sp. B-0618 (FERM-P No. 4049) was inoculated to the culture medium and cultivated at 30° C. for 1 day to obtain a seed culture. Twenty liters of the culture medium having the same composition as that described above were placed in a 30 l volume jar fermenter and sterilized. After transplantation of the seed culture, the medium was cultivated under aeration-agitation at a temperature of 30° C. for 18 hours at 300 r.p.m. and 20 l/min. sterilized air flow rate. The resulting cultivated product was centrifuged to obtain 230 g of wet cells. Then, 230 g of the wet cells were suspended in 3 l of 20 mM phosphate buffer (pH 8.0) containing lysozyme (0.5 mg/ml) and EDTA (2 mM) and the suspension was allowed to react for 1.5 hours at 37° C. under agitation. The suspension was centrifuged at 5,000 r.p.m. for 15 minutes to recover a supernatant (2.85 l, 24,300 U). To the resulting supernatant was added 28.5 ml of a 2% aqueous solution of protamine sulfate and the mixture was centrifuged (5,000 r.p.m., 15 minutes) to obtain a supernatant (2,830 ml, 24,050 U), which was concentrated under reduced pressure (800 ml, 23,600 U). Further, 320 ml of a satu-

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rated ammonium sulfate solution was added to this and the resulting solution was centrifuged at 10,000 r.p.m. for 10 minutes to obtain a supernatant. To this supernatant was added 220 ml of the saturated ammonium sulfate solution. The mixture was centrifuged at 10,000 r.p.m. for 10 minutes to recover precipitates. The precipitates were dissolved in 200 ml of 20 mM phosphate buffer having a pH of 8.0 and the insolubles removed by centrifugation to obtain a supernatant (195 ml, 17,600 U). After the supernatant was further concentrated by ultra filtration (80 ml, 17,400 U), the resulting solution was dialyzed against a 10 mM phosphate buffer (pH 8.0). The solution was charged into diethylaminoethyl agarose ("DEAE-sepharose CL-6B") column (5.0×60 cm) which had been equilibrated with a 10 mM Tris-HCl buffer (pH 8.0). After washing with 2 l of the same buffer containing 0.2 M KCl, the enzyme was eluted with buffers (1.5 l, respectively) containing 0.2 to 0.5 M KCl according to the concentration gradient method, and the active fraction eluted with 0.35-0.37 M KCl was collected (380 ml, 13,500 U). It was concentrated by ultra filtration (40 ml, 13,400 U). The concentrate was charged into acrylamide gel ("Biogel P-2") column for desalting to obtain 90 ml of an eluate (12,500 U). To this was then added 1.8 ml of a 50% sucrose solution and the resulting mixture was lyophilized to obtain 1,265 mg of creatinase powder (specific activity: 8.2 U/mg, recovery: 42.7%).

What we claim is:

1. A method for producing and isolating creatinase which comprises culturing a creatinase producing microorganism *Bacillus* sp. B-0618, Firm-P No. 4049, fractionally eluting sarcosine oxidase and creatinase by anion exchange chromatography from the sarcosine oxidase- and creatinase-containing solution thus produced to obtain a creatinase eluted fraction and collecting creatinase therefrom.

2. A method according to claim 1 wherein the anion exchange chromatography medium is diethylaminoethyl cellulose and the creatinase eluted fraction is eluted with 0.3 M KCl solution in concentration gradient of KCl 0.1 M to 0.5 M.

3. A method according to claim 1 wherein the anion exchange chromatography medium is diethylaminoethyl cellulose and the creatinase eluted fraction is eluted with 0.2-0.5 M KCl solution in concentration gradient of KCl 0.35-0.37 M.

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Purification and Characterization of Creatine Amidinohydrolase of *Alcaligenes* Origin

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Extracellular creatine amidinohydrolase (creatinase, EC 3.5.3.3) produced by *Alcaligenes* sp. nov. was purified to electrophoretic homogeneity by ion exchange chromatography on diethylaminoethyl-cellulose, gel filtration on Sephadex G-75 and hydrophobic chromatography on phenyl-Sepharose CL-4B. The molecular weight of the enzyme was estimated to be 51000 by gel filtration on Sephadex G-200 and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The enzyme showed the maximum activity at pH 8 and was stable at pH 5–9. The pI value was 4.7 as determined by isoelectric focusing. The enzyme catalyzed hydrolysis of creatine to sarcosine and urea, and the K_m and V_{max} values for creatine were 17.2 mM and 105 $\mu\text{mol}/\text{min}/\text{mg}$ protein, respectively. The enzyme was markedly inactivated by *p*-chloromercuribenzoate (PCMB). Besides PCMB, the enzyme was inactivated by *N*-bromosuccinimide, Zn^{2+} , Cu^{2+} or Hg^{2+} .

Keywords—creatin amidinohydrolase (EC 3.5.3.3); creatine; *Alcaligenes*; clinical diagnosis; hydrophobic chromatography

Since the Folin method,¹⁾ which is most commonly used in the clinical diagnostic analysis of creatinine in serum and urine, has the disadvantages of poor specificity and requirement for deproteinization, enzymatic measurement systems^{2–5)} using creatinine amidohydrolase (creatininase, EC 3.5.2.10) and creatine amidinohydrolase (creatinase, EC 3.5.3.3) in combination with other enzymes (for example, sarcosine oxidase and peroxidase) are replacing the Folin method. However, the creatininases and creatinases involved have not yet been characterized completely except for those from *Pseudomonas putida*.

In our screening for creatinine catabolic enzymes, a novel species of *Alcaligenes* was found to be an adequate source for creatinase and creatinase.⁶⁾ The former catalyzes interconversion between creatinine and creatine, and the latter hydrolyzes creatine to sarcosine and urea. The purification and characterization of creatininase produced by *Alcaligenes* have been reported previously.⁷⁾ The present report describes the purification and characterization of creatinase of the same origin.

Materials and Methods

Materials—Sarcosine oxidase (EC 1.5.3.1) of *Bacillus subtilis* origin was obtained from Toyo Jozo Co. and horseradish peroxidase was a product of Wako Chemical Ind., Ltd. Phenyl-Sepharose CL-4B, Sephadex G-75, Sephadex G-200 and Pharmalyte were purchased from Pharmacia Fine Chemicals. All the other materials were commercial products of analytical grade.

Production of Creatinase—A seed culture was established in a 500 ml Sakaguchi flask containing 110 ml of a medium composed of 1.0% soluble starch, 1.0% glucose, 0.75% meat extract, 0.75% polypeptone, 0.3% NaCl, 0.1%

$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001% $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.01% $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.001% $\text{CuSO}_4 \cdot 2\text{H}_2\text{O}$ (pH 7.4) at 28 °C for 72 h on a rotary shaker at 200 rpm. The seed culture (330 ml) was used to inoculate a 10 l jar fermenter (LABOTEC Co., Ltd., LABO LF-100 fermenter) containing 5 l of a production medium consisting of 2.0% glucose, 2.0% soybean meal, 0.5% creatine, 0.2% polypepton, 0.2% NaNO_3 , 0.1% KH_2PO_4 , 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05% KCl and 0.0001% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$. The culture was conducted at 28 °C for 48 h with aeration at 0.8 l/min and agitation at 400 rpm.

Assay for Protein Determination—The protein concentration was measured by the method of Lowry *et al.*⁸⁾ using bovine serum albumin as a standard.

Assay of Creatinase Activity—Assay Method I: A mixture of an aqueous enzyme solution (0.1 ml) and 50 mM Tris-HCl buffer (pH 8.0, 0.9 ml) containing 50 mM creatine was incubated at 37 °C. After 20 min, 2 ml of dimethylsulfoxide containing 1.7% (w/v) *p*-dimethylaminobenzaldehyde and 13% (v/v) conc. HCl was added to produce a yellow dye from urea which was formed by the enzyme reaction. The mixture was allowed to stand at room temperature for 20 min, and the absorbance at 453 nm was measured against the blank, in which the heat-denatured enzyme was employed.

Assay Method II: A mixture of 0.1 M Tris-HCl buffer (pH 8.0, 0.1 ml) and 50 mM creatine (0.8 ml) was warmed to 37 °C and the reaction was started by adding an aqueous enzyme solution (0.1 ml). After incubation for 20 min at 37 °C, the reaction was stopped by adding 1 mM *p*-chloromercuribenzoate (PCMB, 1 ml). The sarcosine formed was determined by further incubation at 37 °C for 20 min with a mixture (0.5 ml) of 30 mM Tris-HCl (pH 8.0), 2.7 mM 4-aminoantipyrine, 0.3 mg/ml phenol, 30 units/ml horseradish peroxidase, 6 units/ml sarcosine oxidase and 0.2 mM PCMB, and the absorbance at 500 nm was read against the blank. In both methods, one unit was defined as the amount of enzyme which catalyzed the hydrolysis of 1 μmol of substrate per min under the conditions described above.

Electrophoresis—Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed in 10% polyacrylamide according to the method of Weber and Osborn.⁹⁾ Bovine serum albumin (M_r 68000), α -chymotrypsinogen (M_r 25700) and lysozyme (M_r 14300) were used as references to determine the molecular weight of the enzyme. Polyacrylamide disc gel electrophoresis was performed according to the method of Davis¹⁰⁾ and specific staining of creatinase activity in the gel was carried out by the formation of diformazan.^{11c)} Isoelectric focusing was conducted according to the method of Låås and Fast-Johansson¹²⁾ using Pharmalyte in a gel rod.

Results

Purification of Creatinase

All the procedures were carried out at 4 °C unless otherwise specified. The culture broth was centrifuged at 3500 rpm for 20 min to remove the mycelial cake. The broth filtrate was brought to 75% saturation with $(\text{NH}_4)_2\text{SO}_4$ at pH 7.8 and allowed to stand for 15 h. The precipitate collected by centrifugation at 3500 rpm for 20 min was dialyzed against 0.02 M Tris-HCl buffer (pH 7.8) for 15 h. Then 2% aqueous protamine sulfate was added to the retentate (0.7 ml/100 mg protein) with stirring and the whole was allowed to stand for 15 h. The supernatant obtained by centrifugation at 3500 rpm for 20 min was dialyzed against 0.02 M Tris-HCl buffer (pH 7.8) for 15 h. The crude sample was concentrated by salting out with 75% saturation of $(\text{NH}_4)_2\text{SO}_4$ and redissolving in 0.02 M Tris-HCl buffer (pH 7.8), and charged on a column (21 cm \times 3.0 cm) of diethylaminoethyl (DEAE)-cellulose (Whatman, DE 23) which had been equilibrated with 0.02 M Tris-HCl buffer (pH 7.8). The column was thoroughly washed with 1 l of the same buffer containing 0.15 M NaCl, then the enzyme was eluted with a linear gradient of NaCl (0.15–0.7 M) in the same buffer. The partially purified enzyme from DEAE-cellulose chromatography was further subjected to Sephadex G-75 column chromatography (Fig. 1) and then to hydrophobic chromatography on phenyl-Sepharose CL-4B (Fig. 2). The purified enzyme was dialyzed against 0.02 M Tris-HCl buffer (pH 7.8) for 15 h and stored in a frozen state for further characterization. The purification of the enzyme is summarized in Table I.

The purified enzyme showed a single band on polyacrylamide gel electrophoresis in the absence or presence of SDS (Fig. 3). On polyacrylamide gel electrophoresis, the relative mobility of the protein band was identical with that of the band stained with diformazan.

Estimation of Molecular Weight

By both SDS-polyacrylamide gel electrophoresis and gel filtration on Sephadex G-200,

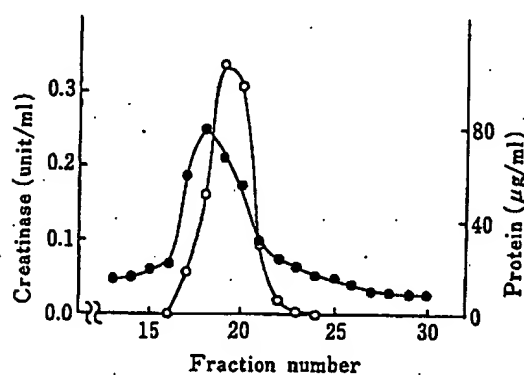


Fig. 1. Gel Filtration of Creatinase on Sephadex G-75

The crude enzyme obtained by DEAE-cellulose chromatography (2.3 mg as protein) was charged on a column of Sephadex G-75 (84 cm \times 1.5 cm), and 10 mM phosphate buffer (pH 7.0) containing 0.1 M NaCl was used for elution of the enzyme. The eluate was collected in 3.8 ml fractions. Creatinase activity was measured by assay method II.

○, creatinase activity; ●, protein.

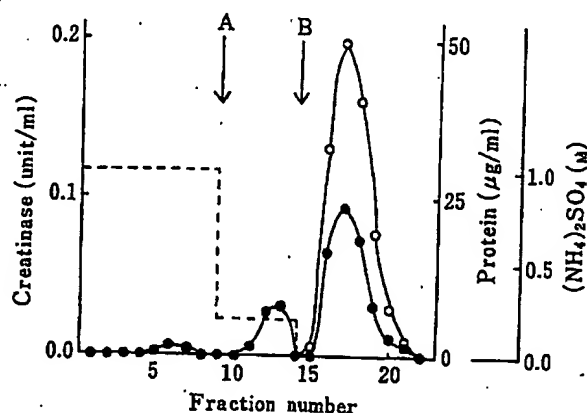


Fig. 2. Hydrophobic Chromatography of Creatinase on Phenyl-Sepharose CL-4B

The peak fractions of creatinase activity from Sephadex G-75 gel filtration were pooled and, after addition of solid $(\text{NH}_4)_2\text{SO}_4$ to a final concentration of 1 M, charged on a column of phenyl-Sepharose CL-4B (5.5 cm \times 1.2 cm) equilibrated with 50 mM phosphate buffer (pH 7.0) containing 1 M $(\text{NH}_4)_2\text{SO}_4$. The column was washed with the same buffer and then 50 mM phosphate buffer (pH 7.0) containing 0.2 M $(\text{NH}_4)_2\text{SO}_4$ (buffer was changed at arrow A). Arrow B indicates a further change to 50 mM phosphate buffer (pH 7.0). The eluate was collected in 2.5 ml fractions and the creatinase activity was measured by assay method II.

○, creatinase activity; ●, protein; ----, $(\text{NH}_4)_2\text{SO}_4$.

TABLE I. Purification of Creatinase

Fraction	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Recovery (%)
Culture broth	49820	3890	0.08	100
Ammonium sulfate ppt.	44850	3690	0.08	95
Protamine sulfate sup.	8580	3350	0.39	86
Ammonium sulfate ppt.	7750	3180	0.41	82
DEAE-cellulose	730	2550	3.49	66
Sephadex G-75	220	1270	5.77	33
Phenyl-Sepharose CL-4B	110	935	8.50	24

a) — [band] +

b) — [band] +

Fig. 3. Electrophorograms of Purified Creatinase

a) Polyacrylamide gel electrophorogram. b) SDS-polyacrylamide gel electrophorogram.

the molecular weight of the enzyme was estimated to be 51000.

Isoelectric Point (pI) of Creatinase

The pI value was determined to be 4.7 by isoelectric focusing.

Effects of pH on Creatinase Activity and Stability

The effects of pH on the enzyme activity and stability were tested by assay methods I and

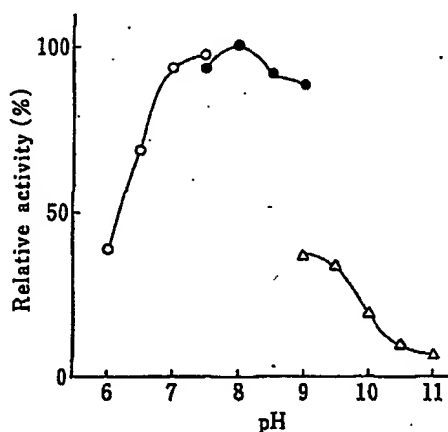


Fig. 4. Effect of pH on Creatinase Activity

The creatinase activity was measured by assay method I. An aqueous enzyme solution (0.39 unit/0.1 ml) was incubated with either 0.1 M phosphate buffer (O, pH 6.0–7.5), 0.1 M Tris-HCl buffer (●, pH 7.5–9.0) or 0.1 M carbonate buffer (Δ, pH 9.0–11.0). The enzyme activity under the standard conditions was defined as 100%.

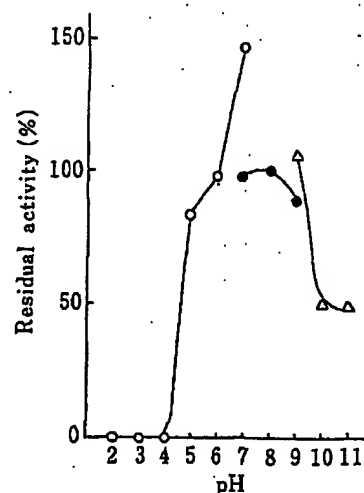


Fig. 5. Effect of pH on the Stability of Creatinase

A mixture of an aqueous enzyme solution (0.063 unit/0.15 ml) and 0.1 M buffer solution (0.15 ml) was incubated at 37°C for 1 h. The pH was adjusted to near 8 with 0.5 M Tris-HCl buffer (pH 8.0, 0.7 ml), then the residual activity was measured by assay method II. The activity of untreated enzyme was defined as 100%.

O, citrate buffer (pH 2.0–7.0); ●, Tris-HCl buffer (pH 7.0–9.0); Δ, carbonate buffer (pH 9.0–11.0).

TABLE II. Effects of Various Chemicals on Creatinase Activity

Chemical (1 mM)	Residual activity (%)
None	100
Ethylethylenediaminetetraacetate	108
8-Hydroxyquinoline	91
<i>o</i> -Phenanthroline hydrochloride	106
Monoiodoacetate	99
<i>p</i> -Chloromercuribenzoate	0
Glutathione (oxidized)	104
<i>p</i> -Toluenesulfonyl chloride	102
Phenylmethanesulfonyl fluoride	99
<i>N</i> -Bromosuccinimide	0

A mixture of the purified enzyme solution (0.276 unit/0.2 ml) in 0.1 M Tris-HCl buffer (pH 8.0) and 5 mM inhibitor solution (0.05 ml) in the same buffer was incubated at 37°C for 30 min and the residual activity was measured by assay method I.

TABLE III. Effects of Various Metal Ions on Creatinase Activity

Metal salt (1 mM)	Residual activity (%)
None	100
MnCl ₂	96
CoCl ₂	77
ZnCl ₂	14
CaCl ₂	98
FeSO ₄	91
MgSO ₄	100
CuSO ₄	51
HgCl ₂	0

A mixture of the purified enzyme solution (0.260 unit/0.2 ml) in 0.1 M Tris-HCl buffer (pH 8.0) and 5 mM aqueous metal salt solution (0.05 ml) was incubated at 37°C for 30 min and the residual activity was measured by assay method I.

II, respectively. The optimum activity was observed near pH 8 (Fig. 4). Although the enzyme was stable at pH 5–9, it seemed especially labile at pH below 4 (Fig. 5).

Effects of Various Chemicals and Metal Ions

The creatinase activity was measured by assay method I. As shown in Table II, the enzyme was completely inactivated by PCMB and *N*-bromosuccinimide. The inhibition by PCMB, however, was reversed by 50% by the further addition of 10 mM dithiothreitol.

Among metal ions tested, the enzyme was adversely affected by Zn^{2+} , Cu^{2+} or Hg^{2+} and negligible effects were observed with the other metal ions (Table III).

Kinetics

Since the enzyme catalyzed the hydrolysis of creatine to sarcosine and urea, the kinetic parameters were obtained from Lineweaver-Burk plots,¹³⁾ using creatine as a substrate. The K_m and V_{max} values were calculated to be 17.2 mM and 105 $\mu\text{mol}/\text{min}/\text{mg}$ protein, respectively, by assay method II, using a reaction mixture composed of an aqueous enzyme solution (0.043 unit/0.1 ml), 0.1 M Tris-HCl buffer (pH 8.0, 0.1 ml) and 2–50 mM aqueous creatine solution (0.8 ml).

Discussion

The creatinase and creatininase were produced by the parental strain of *Alcaligenes* sp. nov. at the ratio of 1:20 in terms of enzymatic activities (0.1 and 2.0 units/ml, respectively). By repeated selections for substrains producing a high titer of creatinase, the productivity of creatinase (0.4 unit/ml) was increased approximately 4 times over that of the parental strain, while the creatininase activity (0.3 unit/ml) was decreased to approximately one-seventh of that of the original strain.

Although creatinyl-AH-Sepharose was used as an affinity adsorbent for the creatininase from *Alcaligenes*, the creatinase could not be separated from impurities which were retained on the column by the strong ionic interaction with guanide groups of the adsorbent. However, hydrophobic chromatography on phenyl-Sepharose CL-4B was found to be effective for the isolation of the creatinase. Furthermore, the creatininase coadsorbed with the creatinase on phenyl-Sepharose CL-4B was not eluted from the column with 50 mM phosphate buffer (pH 7); the enzyme was finally eluted with H_2O .

To date, creatinases from *Arthrobacter ureafaciens*¹⁴⁾ and *Pseudomonas putida*¹¹⁾ have been reported. The former has not yet been purified completely, though the molecular weight was estimated to be 100000. The latter was purified extensively by Yoshimoto *et al.*^{11b,c)} and found to be composed of two subunits with a molecular weight of 47000. Like the creatinase from *Alcaligenes*, the enzyme from *Pseudomonas* was inactivated by PCMB. The pI values of both enzymes are 4.7. However, the creatinase from *Alcaligenes* is a monomer with a molecular weight of 51000. The K_m values for creatine of the enzymes from *Pseudomonas* and *Alcaligenes* are 1.3 and 17.2 mM, respectively. In addition, on polyacrylamide gel electrophoresis, the enzymes showed different mobilities. It is worth emphasizing that the specific activity of the creatinase from *Alcaligenes* is 65 times higher than that of the creatinase from *Pseudomonas*.

The purified enzymes of *Alcaligenes* origin should be useful in the enzymatic determination of creatinine and/or creatine in clinical samples.

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